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APPLICATION

For

UNITED STATES LETTERS PATENT

on

A FAMILY OF TRANSCRIPTIONAL CO-REPRESSORS THAT INTERACT WITH NUCLEAR HORMONE RECEPTORS AND USES THEREFOR

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A Family of Transcriptional Co-repressors that Interact with Nuclear Hormone Receptors and Uses Therefor

RELATED APPLICATIONS

This application is a continuation-in-part application of pending United States application Serial No. 08/522,726, filed September 1, 1995 and is related to United States application Serial No. _____, filed on even date herewith, each of which is incorporated herein in its entirety by reference.

FIELD OF THE INVENTION

The present invention relates to intracellular receptors, methods for the modulation thereof, and methods for the identification of novel ligands therefor. In a particular aspect, the present invention relates to methods for the identification of compounds which function as ligands (or ligand precursors) for intracellular receptors. In another aspect, the present invention relates to novel chimeric constructs and uses therefor.

BACKGROUND OF THE INVENTION

A central problem in eukaryotic molecular biology continues to be the elucidation of molecules and mechanisms that mediate specific gene regulation. As part of the scientific attack on this problem, a great deal of work has been done in efforts to identify ligands (i.e., exogenous inducers) which are capable of mediating specific gene regulation. Additional work has been done in efforts to identify other molecules involved in specific gene regulation.

Although much remains to be learned about the specifics of gene regulation, it is known that ligands modulate gene transcription by acting in concert with intracellular components, including intracellular receptors and discrete DNA sequences known as hormone response elements (HREs).

The identification of compounds that directly or indirectly interact with intracellular receptors, and thereby affect transcription of hormone-responsive genes, would be of significant value, e.g., for therapeutic applications.

Transcriptional silencing mediated by nuclear receptors plays an important role in development, cell differentiation, and is directly linked to the oncogenic activity of v-erbA. The mechanism underlying this effect is unknown but is one key to understanding the molecular basis of hormone action. Accordingly, the identification of components involved in transcriptional silencing would represent a great advance in current understanding of mechanisms that mediate specific gene regulation.

Other information helpful in the understanding and practice of the present invention can be found in commonly assigned United States Patent Nos. 5,071,773, 4,981,784, 5,260,432, and 5,091,513, all of which are hereby incorporated herein by reference in their entirety.

BRIEF DESCRIPTION OF THE INVENTION

The present invention overcomes many problems in the art by providing a family of receptor interacting co-repressors, referred to herein as "SMRT co-repressor", i.e., a silencing mediator (co-repressor) for retinoic acid receptor (RAR) and thyroid hormone receptor (TR). *In vivo*, members of the SMRT family of co-repressors function as potent co-repressors. A GAL4 DNA binding domain (DBD) fusion with a SMRT co-repressor behaves as a frank repressor of a GAL4-dependent reporter.

Together, these observations identify a novel family of cofactors that is believed to represent an important mediator of hormone action.

Accordingly, the present invention provides isolated silencing mediators of retinoic acid and thyroid hormone receptors, and isoforms or peptide portions thereof (SMRT co-repressors), that modulate transcriptional potential of members of the nuclear receptor superfamily. Such SMRT co-repressors comprise a repression domain having less than about 83% identity with a Sin3A interaction domain of N-CoR (amino acids 255 to 312 of SEQ ID NO: 11); less than about 57% identity with repression domain 1 of N-CoR (amino acids 1 to 312 of SEQ ID NO: 11); less than about 66% identity with a SANT domain of N-CoR (amino acids 312 to 668 of SEQ ID NO: 11) and/or; less than about 30% identity with repression domain 2 of N-CoR (amino acids 736 to 1031 of SEQ ID NO: 11).

In accordance with yet another embodiment of the present invention, there are provided isolated peptides comprising at least a portion of the invention SMRT co-repressor six contiguous amino acids of an amino acid sequence selected from the group consisting of:

amino acids 1 to 1030 of SEQ ID NO: 5;
amino acids 1 to 1029 of SEQ ID NO: 7;
amino acids 1 to 809 of SEQ ID NO: 9;
and conservative variations thereof,

provided the peptide is not identical to a sequence of SEQ ID NO: 11.

In addition, there are provided isolated antibodies that bind specifically to invention isolated peptides. There are also provided chimeric molecules comprising invention isolated peptides and at least a second molecule. Also provided are complexes comprising an invention SMRT co-repressor and a member of the superfamily of nuclear receptors and isolated antibodies that bind to such complexes.

Accordingly, the present invention provides isolated polynucleotides encoding members of the newly described family of silencing mediators of retinoic acid and thyroid hormone receptor or an isoform or peptide portion thereof (SMRT co-repressor), or an isolated polynucleotide complementary thereto. In addition, there are provided vectors comprising invention polynucleotides, as well as host cells containing invention polynucleotides.

In additional embodiments of the present invention, there are provided methods for identifying agents that modulate the repressor potential of a SMRT co-repressor.

In another embodiment according to the present invention, there are provided methods for identifying an agent that modulates a function of an invention SMRT co-repressor.

In another embodiment according to the present invention, there are provided methods of modulating the transcriptional potential of a member of the nuclear receptor superfamily (nuclear receptor) in a cell.

In another embodiment according to the present invention, there are provided methods of identifying a molecule that interacts specifically with a SMRT co-repressor.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the quantitation by phosphoimager of a dose-dependent dissociation of SMRT from RAR or TR by all-*trans* retinoic acid (atRA) or thyroid hormone (triiodothyronine or T3).

Figure 2 presents amino acid (aa) sequences of SMRT (Genbank accession number XXXXX). The aa sequence presented in parentheses (i.e., residues

SUB A2 CONT.

1330-1376) is an alternatively spliced insert which is not present in the original two-hybrid clone (C-SMRT, aa 981 to C-terminal end). The proline-rich N-terminal domain (aa 1-160) and the glutamine-rich region (aa 1061-1132), as well as the ERDR and SG regions, are also indicated. The C-terminal region of SMRT (aa 1201 to C-terminal end) shows 48% aa identity to RIP13 (Seol et al., *Molecular Endocrinology* **9**:72-85 (1995)). The rest of the sequence of RIP13 shows 22% aa identity to SMRT (aa 819-1200).

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Figure 3 illustrates mediation of the silencing effect of hRARα and hTRβ by SMRT *in vivo*.

Figure 3(A) illustrates that v-erbA reverses the silencing effect of GAL-RAR (GAL4 DBD-hRARα 156-462) while SMRT restores the silencing effect.

Figure 3(B) illustrates that the RAR403 truncation mutant reverses the silencing effect of GAL-TR (GAL4 DBD-hTRβ 173-456) while SMRT restores the silencing effect.

Figure 3(C) illustrates that v-erbA and full length SMRT or C-SMRT have no effect on GAL-VP16 activity.

Figure 3(D) illustrates that a GAL4 DBD fusion of full length SMRT represses the thymidine kinase basal promoter activity containing four GAL4 binding sites. The fold of repression was calculated by dividing the normalized luciferase activity transfected with the GAL4 DBD alone by those transfected with indicated amount of GAL DBD fusion constructs.

Figure 4 provides an alignment of the human SMRT (SEQ ID NO: 5) and mouse SMRTα (SEQ ID NO: 7) amino acid sequences. Proteins were aligned using the CLUSTAL alignment program. Underlined sequence of mouse SMRTα corresponds to the amino acid sequences that are deleted in mouse SMRTβ. The

arrow indicates the start point of the previously described human SMRT co-repressor (sSMRT).

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Figures 5A and 5B provide alignments of the human SMRT and human N-CoR co-repressors.

Figure 6A is a graph showing the results of transactivation experiments using transcripts encoding a detectable reporter and either wild type EcR (Ecr wt), a repression-Defective EcR allele Ecraa^{483T} (EcRA483T) or vp16 activation domain fused to Ultraspiracle (vp16-USP).

Figure 6B is a graph showing the results of transactivation experiments using CMV promoter-driven expression vectors. Wild-type EcR or EcR A483T was cotransfected with vp16-USP and Gal4-c-SMRT (aa 981 to C terminus) (Chen and Evans, *Nature* 377:454-457, (1995)) into CV-1 cells to examine its effect on the interaction with vertebrate corepressor. All cells were also cotransfected with a TK-luciferase reporter construct, pMH100-TK-Luc, containing four copies of the yeast Gal4-responsive element.

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Figure 6C shows alignment of EcR, rTR, hRAR, and rRev-erbA receptor sequences and the secondary structure in the LBD signature motif region. Conserved residues are marked in dark. The mutation 483 (AT) is marked at the top of the corresponding residue.

Figure 7 is a graph showing β-galactosidase activity in a yeast two-hybrid screen with pAS-EcR as bait. pAS-EcR is a fusion gene with the region corresponding to aa 223-878 of EcRB1 fused C-terminally to the Gal4-DBD of the pAS1-CYH2 construct (Durfee et al., *Genes Dev* 7:555-569 (1993)); other Gal4-DBD-based nuclear receptor constructs used in this yeast two-hybrid assay include: USP (aa 50-508), hRAR (aa 186-462) and hTR (aa 121-410) (Schulman et al., *Proc. Natl. Acad. Sci. USA*, 92:8288-8292, (1995)), and SMRT (Chen and Evans, (1995), *supra*).

β-galactosidase activities were quantified by liquid assay for yeast cells treated either without ligand or with 3 μM of corresponding hormone. All-trans retinoic acid (ATRA) is a ligand of RAR; 3,3',5-triiodothyroacetic acid (T3) is a ligand of TR. RAR, retinoic acid receptor; TR, thyroid hormone receptor.

Figure 8A shows the complete amino acid sequence of the SMRTER protein (SEQ ID NO: 12). The underlined regions represent the residues also conserved in SMRT and N-CoR. The gray box indicates the sequences of the E52 clone.

Figure 8B is a schematic structural diagram of SMRTER, SMRT, and N-CoR showing the conserved SNOR, SANT, GST, ITS, D/ER repeat, and LSD motifs with their designated patterns positioned in their relative regions in each protein.

Figure 9. Sequence Comparison of SMRTER, SMRT, N-CoR, and Other Related Proteins. The SANT domains of various proteins are listed. Percent identities/similarities compared to SMRTER are shown on the right. Two potential helices are predicted in the N-terminal half of the SANT domain. Black boxes indicate identical sequences; gray boxes, similar or partially identical sequences.

Figure 10 is a schematic representation showing functional domains in SMRTER. Numbers on the left represent the regions in SMRTER used to generate the Gal4-DBD fusion genes. Black stippled bars indicate the locations of EcR-interacting domains; gray stippled bars indicate repression domains. Plus signs indicate that a positive interaction between SMRTER and the EcR complex and repression of basal activity by Gal4-SMRTER is significant. ERID = ecdysone receptor-interacting domain; SMRD = SMRTER repressor domain.

Figure 11A is a graph showing the interaction of ERID1 AND ERID2 with the EcR complex. Figure 11B is a graph showing the results of competition

between ERID1, ERID2 and c-SMRT for binding to EcR. Figure 11C is a graph showing that EcR A483T disrupts the interaction with ERID1 and ERID2.

Figure 12A shows the results of mapping three repression domains. To examine repressive activity, transcriptional activity of each Gal4-SMRTER fusion was compared to the basal activity of Gal4-DBD on reporter. Only repression with value approximately 5-fold or over is considered positive (+).

Figure 12B is a schematic representation of mapping the SMRTER-interacting domain in mSin3A and dSin3A. Yeast two-hybrid assays were used to assess the interaction between each Gal4-DBD-based fusion gene of each SMRD and the ACT-based fusion genes of mSin3A and dSin3A. The numbers indicate the region in either mSin3A or in dSin3A used to generate the ACT fusion genes. Constructs of mSin3A were described previously in Nagy et al., *Cell* 89:373-380, (1997).

Figure 12C shows an alignment of SMRD3 of SMRTER and an mSin3-interacting domain of N-CoR. Conserved residues are boxed in gray. An asterisk indicates the region where the mutation (Gly) was generated. Minus signs indicate that the interaction between SMRD3 and Sin3A was not detectable in the yeast two-hybrid assays. Repression was measured by comparing the transcriptional activity of Gal4-SMRD3 M2 or Gal4-SMRD3 M3 to that of wild-type Gal4-SMRD3 using transfection experiments as described above.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a family of isolated SMRT co-repressors, and isoforms and peptide portions thereof, that modulate transcriptional potential of members of the nuclear receptor superfamily. Exemplary members of this family are co-repressors having substantially the same sequence as residues 1-1329 plus 1376-1495, as set forth in SEQ ID NO:1, optionally further

comprising the amino acid residues set forth in SEQ ID NO:2 (i. e., residues 1330-1375 of SEQ ID NO:1).

In another embodiment according to the present invention, the invention SMRT co-repressor comprises a repression domain having less than about 83% identity with a Sin3A interaction domain of N-CoR (as amino acids 255 to 312 of SEQ ID NO: 11); less than about 57% identity with repression domain 1 of N-CoR (amino acids 1 to 312 of SEQ ID NO: 11); less than about 66% identity with a SANT domain of N-CoR (amino acids 312 to 668 of SEQ ID NO: 11 and/or; less than about 30% identity with repression domain 2 of N-CoR (amino acids 736 to 1031 of SEQ ID NO: 11). Such an encoded SMRT co-repressor or peptide portion thereof is further characterized in that it can modulate transcriptional potential of a member of the nuclear receptor superfamily (nuclear receptor).

The invention SMRT co-repressors are additionally exemplified by a full length human SMRT co-repressor, (amino acids 1 to 2517 of SEQ ID NO: 5); and by two mouse SMRT isoforms, including a longer SMRT isoform designated mouse SMRTα, which has an amino acid sequence set forth as amino acids 1 to 2473 of SEQ ID NO: 7; and a shorter SMRT isoform designated mouse SMRTβ (amino acids 1 to 2253 of SEQ ID NO: 9). As compared to the mouse SMRTα isoform (SEQ ID NO: 7), the mouse SMRTβ isoform (SEQ ID NO: 9) has a deletion corresponding to amino acids 36 to 254 of SEQ ID NO: 7.

A peptide portion of a SMRT co-repressor is exemplified herein by amino acids 1 to 1031 of SEQ ID NO: 5; amino acids 1 to 1031 of SEQ ID NO: 7; and amino acids 1 to 813 of SEQ ID NO: 9, which includes the entire amino terminal domain of a SMRT co-repressor. Additional peptide portions of a SMRT co-repressor are exemplified by amino acids 1 to 303 of SEQ ID NO: 7; amino acids 845 to 986 of SEQ ID NO: 7; amino acids 427 to 663 of SEQ ID NO: 7; amino acids 845 to 1055 of SEQ ID NO: 7; amino acids 736 to 1031 of SEQ ID NO: 7; and amino acids 1 to 85 of SEQ ID NO: 9, which are sub-domains of the amino terminal domain

of mouse SMRTα that have nuclear receptor repressor potential, as well as by the corresponding peptide portions of human SMRT and corresponding peptide portions of mouse SMRTβ, which can modulate the transcriptional potential of a nuclear receptor, particularly a nuclear receptor that is in the form of a dimer, for example, a thyroid hormone receptor homodimer, a retinoic acid receptor homodimer, a retinoid X receptor homodimer, a thyroid hormone receptor-retinoid X receptor heterodimer, or a retinoic acid receptor-retinoid X receptor heterodimer. In addition, the invention relates to isolated peptides that contain at least six contiguous amino acids of an amino acid sequence set forth as amino acids 1 to 1030 of SEQ ID NO: 5; amino acids 1 to 1029 of SEQ ID NO: 5; or amino acids 1 to 809 of SEQ ID NO: 9, provided the SMRT peptide is not identical to a sequence of N-CoR (SEQ ID NO: 11).

Invention co-repressor can be an invertebrate SMRT co-repressor, such as the Drosophilia SMRTER co-repressor having an amino acid sequence as set forth in SEQ ID NO: 12, or conservative variations thereof.

Additional exemplary co-repressors are those containing one or both of the receptor interacting domains (ERID1 and ERID2) identified in the Drosophilia co-repressor. For example, co-repressors containing such receptor interacting domains can be selected from the following segments of the Drosophilia SMRTER co-repressor (SEQ. ID 12):

amino acids 1698-1924 of SEQ. ID NO:12,
amino acids 2951-3038 of SEQ. ID NO:12,
amino acids 1698-2063 of SEQ. ID NO:12,
amino acids 2094-3040 of SEQ. ID NO:12,
amino acids 2929-3181 of SEQ. ID NO:12,
amino acids 542-950 of SEQ. ID NO:12,
amino acids 2094-3181 of SEQ ID NO:12,
amino acids 2929-3040 of SEQ ID NO:12, and

amino acids 2951-3038 of SEQ ID NO:12,
and conservative variations thereof.

Additional exemplary co-repressors are those containing one or more of three autonomous repressor domains termed SMRD1, SMRD2, and SMRD3 identified in the SMRTER co-repressor. For example, invention co-repressors can contain the following autonomous repressor domains derived from Drosophilia SMRTER co-repressor (SEQ. ID 12):

amino acids 542-950 of SEQ. ID NO:12
amino acids 1698-1924 of SEQ ID NO:12,
amino acids 2951-3038 of SEQ. ID NO:12, and conservative variations thereof.

Conservative variations of the above-described SMRT co-repressors are also contemplated to be within the scope of the present invention. Moreover, proteins, polypeptides and peptides having at least 80% sequence identity with any of the SMRT co-repressors described herein are also contemplated to be within the scope of the invention.

In another embodiment according to the present invention, there are provided chimeric molecules comprising invention isolated peptides and at least a second molecule. For example, the second molecule in invention chimeric molecule can be a polynucleotide or a polypeptide. In one embodiment, the chimeric molecule is a fusion polypeptide comprising a SMRT co-repressor operably linked to a DNA binding domain of a transcription factor.

In another embodiment according to the present invention, there are provided isolated antibodies that bind specifically to invention isolated peptides. In one embodiment, an antibody of the invention binds specifically to an epitope of a SMRT co-repressor. Such an antibody is characterized, in part, in that it does not substantially crossreact with an N-CoR polypeptide. In another embodiment, an

antibody of the invention binds specifically to a complex, which includes a SMRT co-repressor or peptide portion thereof of the invention, a nuclear receptor and, optionally, a DNA regulatory element that is specifically bound by the nuclear receptor. Such an antibody is characterized, in part, in that it does not substantially crossreact with the nuclear receptor, either alone or bound to the DNA regulatory element. An antibody of the invention can be a monoclonal antibody, or can be one of a plurality of polyclonal antibodies, which essentially is a mixed population of monoclonal antibodies. The invention also relates to a cell line, which produces the monoclonal antibody of the invention.

Such antibodies can be employed for a variety of purposes, e.g., for studying tissue localization of invention SMRT co-repressor, the structure of functional domains, the purification of receptors, as well as in diagnostic applications, therapeutic applications, and the like. Preferably, for therapeutic applications, the antibodies employed will be monoclonal antibodies.

The above-described antibodies can be prepared employing standard techniques, as are well known to those of skill in the art, using the invention SMRT co-repressor or portions thereof as antigens for antibody production. Both anti-peptide and anti-fusion protein antibodies can be used [see, for example, Bahouth et al. (1991) Trends Pharmacol Sci. vol. 12:338-343; Current Protocols in Molecular Biology (Ausubel et al., eds.) John Wiley and Sons, New York (1989). Factors to consider in selecting portions of invention SMRT co-repressor for use as immunogen (as either a synthetic peptide or a recombinantly produced bacterial fusion protein) include antigenicity, accessibility (i.e., where the selected portion is derived from, e.g., the ligand binding domain, DNA binding domain, dimerization domain, and the like), uniqueness of the particular portion selected (relative to known receptors and co-repressors therefor), and the like.

In another embodiment according to the present invention, there are provided complexes comprising an invention SMRT co-repressor and a member of

the nuclear receptor superfamily and isolated antibodies that bind to such complexes. The nuclear receptor can be in the form of a monomer or dimer, for example, a thyroid hormone receptor homodimer, a retinoic acid receptor homodimer, a retinoid X receptor homodimer, a thyroid hormone receptor-retinoid X receptor heterodimer, a retinoic acid receptor-retinoid X receptor heterodimer, a ecdysone receptor-Ultraspiracle receptor heterodimer, and the like. Optionally or alternatively, the complex can include a DNA regulatory element, bound specifically by a DNA binding domain of the nuclear receptor.

The above-described complexes optionally further comprise a response element for the member of the nuclear receptor superfamily. Such response elements are well known in the art. Thus, for example, RAR response elements are composed of at least one direct repeat of two or more half sites separated by a spacer of five nucleotides. The spacer nucleotides can independently be selected from any one of A, C, G or T. Each half site of response elements contemplated for use in the practice of the invention comprises the sequence

-RGBNNM-,

wherein

R is selected from A or G;

B is selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

M is selected from A or C;

with the proviso that at least 4 nucleotides of said -RGBNNM- sequence are identical with the nucleotides at corresponding positions of the sequence -AGGTCA-. Response elements employed in the practice of the present invention can optionally be preceded by N_x, wherein x falls in the range of 0 up to 5.

Similarly, TR response elements can be composed of the same half site repeats, with a spacer of four nucleotides. Alternatively, palindromic constructs as have been described in the art are also functional as TR response elements.

The above-described SMRT co-repressor/dimeric receptor complexes can be dissociated by contacting the complex with a ligand for the member of the nuclear receptor superfamily.

As employed herein, the term "ligand (or ligand precursor) for a member of the nuclear receptor superfamily" (i.e., intracellular receptor) refers to a substance or compound which, in its unmodified form (or after conversion to its "active" form), inside a cell, binds to receptor protein, thereby creating a ligand/receptor complex, which in turn can activate an appropriate hormone response element. A ligand therefore is a compound which acts to modulate gene transcription for a gene maintained under the control of a hormone response element, and includes compounds such as hormones, growth substances, non-hormone compounds that modulate growth, and the like. Ligands include steroid or steroid-like hormone, retinoids, thyroid hormones, pharmaceutically active compounds, and the like. Individual ligands may have the ability to bind to multiple receptors.

Accordingly, as employed herein, "putative ligand" (also referred to as "test compound") refers to compounds such as steroid or steroid-like hormones, pharmaceutically active compounds, and the like, that are suspected to have the ability to bind to the receptor of interest, and to modulate transcription of genes maintained under the control of response elements recognized by such receptor.

In another embodiment according to the present invention, there are provided polynucleotides encoding members of the above-described family of silencing mediators of retinoic acid and thyroid hormone receptor, or an isoform or peptide portion thereof (SMRT co-repressors), or an isolated polynucleotide complementary thereto.

Invention polynucleotides include those encoding a SMRT co-repressor comprises a repression domain having

a) less than about 83% identity with a Sin3A interaction domain of N-CoR set forth as amino acids 255 to 312 of SEQ ID NO: 11;

b) less than about 57% identity with repression domain 1 of N-CoR set forth as amino acids 1 to 312 of SEQ ID NO: 11;

c) less than about 66% identity with a SANT domain of N-CoR set forth as amino acids 312 to 668 of SEQ ID NO: 11; or

d) less than about 30% identity with repression domain 2 of N-CoR set forth as amino acids 736 to 1031 of SEQ ID NO: 11.

In addition, an invention polynucleotide can encode a mouse SMRTβ isoform having an amino acid sequence as set forth in SEQ ID NO: 9 or conservative variations thereof, or a polynucleotide having a nucleotide sequence as set forth in SEQ ID NO: 8.

Further examples of invention polynucleotides are those comprising a nucleotide sequence selected from the group consisting of:

nucleotides 1 to 3094 of SEQ ID NO: 4;

nucleotides 1 to 3718 of SEQ ID NO: 6;

nucleotides 1 to 2801 of SEQ ID NO: 8;

nucleotides 1 to 8388 of SEQ ID NO: 6;

nucleotides 1 to 7465 of SEQ ID NO: 8; and

nucleotides 1 to 8561 of SEQ ID NO: 4.

The invention polynucleotides further comprise those encoding a human SMRT co-repressor having an amino acid sequence as set forth in SEQ ID NO: 5, for example, a nucleotide sequence as set forth in SEQ ID NO: 4; by a polynucleotide encoding a mouse SMRTα isoform having an amino acid sequence as set forth in SEQ ID NO: 7, for example, a nucleotide sequence as set forth in SEQ ID NO: 6; and by a polynucleotide encoding a mouse SMRTβ isoform having an amino acid sequence as set forth in SEQ ID NO: 9, for example, a nucleotide sequence as set forth in SEQ ID NO: 8. A polynucleotide of the invention is further exemplified by

polynucleotides encoding peptide portions of a SMRT co-repressor such as a polynucleotide containing nucleotides 1 to 3094 of SEQ ID NO: 4; nucleotides 1 to 3718 of SEQ ID NO: 7; or nucleotides 1 to 2801 of SEQ ID NO: 8, which can repress the transcriptional activity of nuclear receptor, particularly a nuclear receptor that is in the form of dimer.

Additional invention polynucleotides include those encoding a full length insect SMRTER co-repressor having an amino acid sequence as set forth in SEQ ID NO: 12, or conservative variations thereof.

Additional exemplary invention polynucleotides are those encoding one or both of the receptor interacting domains (ERID1 and ERID2) identified in invention co-repressors. For example, polynucleotides encoding such receptor interacting domains can be selected from those encoding the following segments of the Drosophilia SMRTER co-repressor (SEQ. ID 12):

amino acids 1698-1924 of SEQ. ID NO:12,
amino acids 2951-3038 of SEQ. ID NO:12,
amino acids 1698-2063 of SEQ. ID NO:12,
amino acids 2094-3040 of SEQ. ID NO:12,
amino acids 2929-3181 of SEQ. ID NO:12,
amino acids 542-950 of SEQ. ID NO:12,
amino acids 2094-3181 of SEQ ID NO:12,
amino acids 2929-3040 of SEQ ID NO:12, and
amino acids 2951-3038 of SEQ ID NO:12,
and conservative variations thereof.

Additional exemplary invention polynucleotides are those encoding one or more of three autonomous repressor domains termed SMRD1, SMRD2, and SMRD3 identified in the invention co-repressors. For example, polynucleotides encoding such autonomous repressor domains can be selected from those encoding the following segments of the Drosophilia SMRTER co-repressor (SEQ. ID 12):

amino acids 542-950 of SEQ. ID NO:12

amino acids 1698-1924 of SEQ ID NO:12,

amino acids 2951-3038 of SEQ. ID NO:12, and conservative variations thereof.

A polynucleotide that has at least 80% sequence identity or that hybridizes, (preferably under high stringency conditions) with any one of the above-described polynucleotides is also contemplated to be within the scope of this invention.

A polynucleotide of the invention can be operably linked to a second nucleotide sequence and, therefore, can encode a fusion polypeptide, for example, a SMRT co-repressor, or peptide portion thereof, operably linked to a DNA binding domain of a transcription factor.

Additional examples of invention isolated oligonucleotides, are those which generally are at least about 15 nucleotides in length and can hybridize specifically to the polynucleotide of the invention, but not to a polynucleotide encoding an N-CoR polypeptide (SEQ ID NO: 11). An oligonucleotide of the invention can be useful as a probe, or as a primer for a PCR procedure, or can encode a peptide containing at least five contiguous amino acids of a SMRT co-repressor. In one embodiment, an oligonucleotide of the invention encodes at least five contiguous amino acids of a sequence such as that shown as amino acids 720 to 745 of SEQ ID NO: 5; or amino acids 716 to 742 of SEQ ID NO: 7; or amino acids 497 to 523 of SEQ ID NO: 9. In another embodiment, an oligonucleotide of the invention can hybridize specifically to a polynucleotide encoding human SMRT (SEQ ID NO: 5) or mouse SMRTα (SEQ ID NO: 7), and, optionally, to a polynucleotide encoding mouse SMRTβ (SEQ ID NO: 9).

The phrase "substantially the same" as used herein in reference to a nucleotide sequence of DNA, a ribonucleotide sequence of RNA, or an amino acid

sequence of protein, means sequences that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species that are substantially the same are considered to be equivalent to the disclosed sequences and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" means that sequences substantially the same as the DNA, RNA, or proteins disclosed and claimed herein are functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

In another embodiment according to the present invention, there are provided vectors comprising an invention polynucleotide, and host cells containing invention polynucleotides. The invention vector can be an expression vector, including, for example, a viral vector, and the polynucleotide, or a vector containing the polynucleotide, can be contained in a host cell. In one embodiment, the polynucleotide of the invention is operably linked to a tissue specific DNA regulatory element. In another embodiment, a SMRT co-repressor or peptide portion thereof encoded by the polynucleotide is expressed in a host cell.

In another embodiment according to the present invention, there are provided methods for identifying an agent that modulates the repressor potential of a SMRT co-repressor. In this embodiment, the invention method comprises contacting a host cell with an agent, and detecting a change in the level of expression of a first expressible nucleotide sequence in response to the agent, thereby identifying an agent that modulates the repressor potential of a SMRT co-repressor. In such a method, the host cell is characterized, in part, in that it contains a first expressible nucleotide

sequence operably linked to a first DNA regulatory element, and expresses a fusion polypeptide composed of an invention SMRT co-repressor, or peptide portion thereof, and a DNA binding domain of a first transcription factor that can specifically bind the first DNA regulatory element. Binding of the DNA binding domain of the first transcription factor to the first DNA regulatory element results in expression of the first expressible nucleotide sequence in the host cell.

In another embodiment according to the present invention, there are provided methods for identifying an agent that modulates a function of an invention SMRT co-repressor. In this embodiment, the invention method comprises contacting an invention SMRT co-repressor, a member of the nuclear receptor superfamily, and an agent, and detecting an altered activity of the SMRT co-repressor in the presence of the agent as compared to the absence of the agent, thereby identifying an agent that modulates a function of the SMRT co-repressor.

A method of the invention can be performed, for example, by contacting a host cell with an agent, and detecting a change in the level of expression of a first expressible nucleotide sequence in response to the agent, thereby identifying an agent that modulates the repressor potential of a SMRT co-repressor. In such a method, the host cell is characterized, in part, in that it contains a first expressible nucleotide sequence operably linked to a first DNA regulatory element, and expresses a fusion polypeptide composed of a SMRT co-repressor or peptide portion thereof of the invention, and a DNA binding domain of a first transcription factor, which can specifically bind the first DNA regulatory element; binding of the DNA binding domain of the first transcription factor to the first DNA regulatory element results in expression of the first expressible nucleotide sequence in the host cell. The first expressible nucleotide sequence can be an endogenous gene, which is normally present in the host cell, or can be a sequence that has been introduced into the host cell, either transiently or stably, using methods of recombinant DNA technology. In one embodiment, the first DNA binding domain is a GAL4 DNA binding domain and the first DNA regulatory element is a GAL4 DNA regulatory element that is operably

linked to an expressible nucleotide sequence, for example, a reporter gene, and is introduced into the host cell.

Thus, the invention method can identify an agent that increases or decreases the repressor potential of the SMRT co-repressor, or of an agent that increases or decreases the function of the SMRT co-repressor. The agent can directly interact with the SMRT co-repressor or peptide portion thereof, thereby modulating the repressor potential or function of the SMRT co-repressor, or can interact with a cellular molecule that, in turn, can alter the repressor potential or function of a SMRT co-repressor, thereby increasing or decreasing the repressor potential of the SMRT co-repressor.

The host cell can optionally contain a second expressible nucleotide sequence operably linked to a second DNA regulatory element, and can express a second fusion polypeptide, which is composed of an N-CoR polypeptide, or a repressor domain thereof, and a DNA binding domain of a second transcription factor, which can specifically bind the second DNA regulatory element. By comparing the level of expression of the first expressible nucleotide sequence and the second expressible nucleotide sequence in the host cell upon contacting the host cell with the agent, an agent that independently or coordinately modulates SMRT and N-CoR repressor activity. For example, detecting a change in the level of expression of the first expressible nucleotide sequence, but not in the level of expression of the second expressible nucleotide sequence, due to contacting the host cell with the agent identifies an agent that modulates the repressor potential of a SMRT co-repressor, but not of an N-CoR polypeptide can be identified.

In practicing a method of the invention, the SMRT co-repressor, or peptide portion thereof, can be, for example, an amino acid sequence such as amino acids 1 to 1031 of SEQ ID NO: 5; amino acids 1 to 1031 of SEQ ID NO: 7; or amino acids 1 to 813 of SEQ ID NO: 9. The agent can be, for example, an antibody or antigen binding fragment thereof, a peptide, or a small organic molecule.

In another embodiment according to the present invention, there are provided methods of modulating the transcriptional potential of a member of the nuclear receptor superfamily (nuclear receptor) in a cell, the method comprising introducing an invention isolated polynucleotide into the cell, whereby the polynucleotide or an expression product of the polynucleotide alters the level of a SMRT co-repressor in the cell, thereby modulating the transcriptional potential of the nuclear receptor.

In another embodiment according to the present invention, there are provided methods of modulating the transcriptional potential of a member of the nuclear receptor superfamily (nuclear receptor) in a cell, the method comprising introducing an invention isolated polynucleotide into the cell, whereby the polynucleotide or an expression product of the polynucleotide alters the level of a SMRT co-repressor in the cell, thereby modulating the transcriptional potential of the nuclear receptor.

In performing a method of the invention, an agent that alters an interaction of the SMRT co-repressor, or peptide portion thereof, with the nuclear receptor can be identified using a binding assay, such as an electrophoretic mobility shift assay wherein the level of expression of an expressible nucleotide sequence. Such a method can also identify an agent that alters the ability of the invention SMRT co-repressor, or peptide portion thereof, to interact specifically with the nuclear receptor, but does not alter the level of expression of the expressible nucleotide sequence; or an agent that alters the level of expression of the expressible nucleotide sequence, but does not alter interaction of the SMRT co-repressor or peptide portion thereof with the nuclear receptor; or an agent that alters an interaction of the SMRT co-repressor, or peptide portion thereof, with the nuclear receptor and alters the level of expression of the expressible nucleotide sequence. The agent can, but need not be, a ligand for the nuclear receptor, and the method can be performed in a cell or in a reaction mixture *in vitro*.

Alternatively, an invention polynucleotide can be introduced into the cell, whereby the polynucleotide, or an expression product of the polynucleotide, alters the level of a SMRT co-repressor in the cell, thereby modulating the transcriptional potential of the nuclear receptor. The polynucleotide can encode an invention SMRT co-repressor or peptide, portion thereof, which can be expressed in the cell, thereby increasing the level of a SMRT co-repressor, or peptide portion thereof, in the cell. The polynucleotide also can be an antisense polynucleotide, that decreases the level of a SMRT co-repressor in the cell.

In another embodiment according to the present invention, there are provided methods of identifying a molecule that interacts specifically with a SMRT co-repressor. In this embodiment, invention methods comprise contacting the molecule with an invention SMRT co-repressor and detecting specific binding of the molecule to the SMRT co-repressor, thereby identifying a molecule that interacts specifically with a SMRT co-repressor.

The molecule can be any molecule that interacts specifically with a SMRT co-repressor, including, for example, a small organic molecule such as a drug, a peptide, a nucleic acid molecule, and the like. In one embodiment, the molecule is a cellular factor, for example, a cellular protein that modulates the ability of a SMRT co-repressor to repress transcriptional activity of a nuclear receptor. In another embodiment, the method further involves isolating the molecule that interacts specifically with the SMRT co-repressor or peptide portion thereof.

In accordance with yet another aspect of the present invention, there are provided methods to block the repressing effect of invention SMRT co-repressors, said method comprising administering an effective amount of an antibody as described herein. Alternatively, a silencing domain of a nuclear receptor can be employed. Those of skill in the art can readily determine suitable methods for administering said antibodies, and suitable quantities for administration, which will vary depending on

numerous factors, such as the indication being treated, the condition of the subject, and the like.

In accordance with another aspect of the present invention, there is provided a method to repress (or silence) the activity of a member of the nuclear receptor superfamily containing a silencing domain that represses basal level promoter activity of target genes, said method comprising contacting said member of the nuclear receptor superfamily with a sufficient quantity of an invention SMRT co-repressor so as to repress the activity of said member. Members of the nuclear receptor superfamily contemplated for repression in accordance with this aspect of the present invention include, for example, thyroid hormone receptor, retinoic acid receptor, vitamin D receptor, peroxisome proliferator activated receptor, and the like.

In accordance with yet another aspect of the present invention, there is provided a method to identify compounds which relieve the repression of nuclear receptor activity caused by an invention SMRT co-repressor, said method comprising comparing the size of the SMRT co-repressor/dimeric receptor complex (i.e., complexes comprising the invention SMRT co-repressor and a homodimeric or heterodimeric member of the nuclear receptor superfamily) upon exposure to test compound, relative to the size of said complex in the absence of test compound. An observed size corresponding to intact complex is indicative of an inactive compound, while an observed size that reflects dissociation of the complex is indicative of a compound that disrupts the complex, thereby relieving the repression caused thereby. Optionally, the complex employed in this assay further comprises a response element for said member of the nuclear receptor superfamily.

The size of the above-described complex can readily be determined employing various techniques available in the art. For example, electrophoretic mobility shift assays (EMSA) can be employed (wherein receptor alone or receptor-SMRT co-repressor complex is bound to target DNA and the relative mobility thereof determined).

Those of skill in the art can readily identify other methodology which can be employed to determine the size of the complex as a result of exposure to putative ligand.

In accordance with a still further aspect of the present invention, there is provided a method to identify compounds which relieve the repression of nuclear receptor activity caused by an invention SMRT co-repressor, without substantially activating said receptor, said method comprising:

comparing the reporter signal produced by two different expression systems in the absence and presence of test compound,

wherein said first expression system comprises a complex comprising:

a homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer, or retinoic acid receptor-retinoid X receptor heterodimer,

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a reporter gene, and

optionally, invention SMRT co-repressor, and

wherein said second expression system comprises a complex comprising:

a homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first expression system, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes,

the same response element-reporter combination as employed in said first expression system, and

optionally, invention SMRT co-repressor, and thereafter selecting those compounds which provide:

a higher reporter signal upon exposure of said compound to said first expression system, relative to reporter signal in the absence of said compound, and

substantially the same reporter signal upon exposure of said compound to said second expression system, relative to reporter signal in the absence of said compound,

wherein said selected compounds are capable of relieving the repression of nuclear receptor activity caused by a SMRT co-repressor having a structure and function characteristic of an invention SMRT co-suppressor but substantially lacking the ability to activate nuclear receptor activity.

The addition of invention SMRT co-repressor is optional in the above-described assay because it is present endogenously in most host cells employed for such assays. It is preferred, to ensure the presence of a fairly constant amount of SMRT co-repressor, and to ensure that SMRT co-repressor is not a limiting reagent, that SMRT co-repressor be supplied exogenously to the above-described assays.

Mutant receptors contemplated for use in the practice of the present invention are conveniently produced by expression plasmids, introduced into the host cell by transfection. Mutant receptors contemplated for use herein include RAR403 homodimers, RAR403-containing heterodimers, TR160 homodimers, TR160-containing heterodimers, and the like.

Reporter constructs contemplated for use in the practice of the present invention comprise:

(a) a promoter that is operable in the host cell,

(b) a hormone response element, and

(c) a DNA segment encoding a reporter protein,

wherein the reporter protein-encoding DNA segment is operatively linked to the promoter for transcription of the DNA segment, and

wherein the hormone response element is operatively linked to the promoter for activation thereof.

Hormone response elements contemplated for use in the practice of the present invention are well known in the art, as has been noted previously.

Exemplary reporter genes include chloramphenicol transferase (CAT), luciferase (LUC), beta-galactosidase (β -gal), and the like. Exemplary promoters include the simian virus (SV) promoter or modified form thereof (e.g., SV), the thymidine kinase (TK) promoter, the mammary tumor virus (MTV) promoter or modified form thereof (e.g., ΔMTV), and the like [see, for example, Mangelsdorf et al., in Nature 345:224-229 (1990), Mangelsdorf et al., in Cell 66:555-561 (1991), and Berger et al., in J. Steroid Biochem. Molec. Biol. 41:733-738 (1992).

As used herein in the phrase "operative response element" or "operatively linked" the word "operative" means that the respective DNA sequences (represented by the terms "GAL4 response element" and "reporter gene") are operational, i.e., work for their intended purposes; such that after the two segments are linked, upon appropriate activation by a ligand-receptor complex, the reporter gene will be expressed as the result of the fact that the "GAL4 response element" was "turned on" or otherwise activated.

In practicing the above-described functional bioassay, the expression plasmid and the reporter plasmid are co-transfected into suitable host cells. The transfected host cells are then cultured in the presence and absence of a test compound to determine if the test compound is able to produce activation of the promoter operatively linked to the response element of the reporter plasmid. Thereafter, the transfected and

cultured host cells are monitored for induction (i.e., the presence) of the product of the reporter gene sequence.

Any cell line can be used as a suitable "host" for the functional bioassay contemplated for use in the practice of the present invention. Thus, cells contemplated for use in the practice of the present invention include transformed cells, non-transformed cells, neoplastic cells, primary cultures of different cell types, and the like. Exemplary cells which can be employed in the practice of the present invention include Schneider cells, CV-1 cells, HuTu80 cells, F9 cells, NTERA2 cells, NB4 cells, HL-60 cells, 293 cells, Hela cells, yeast cells, and the like. Preferred host cells for use in the functional bioassay system are COS cells and CV-1 cells. COS-1 (referred to as COS) cells are monkey kidney cells that express SV40 T antigen (Tag); while CV-1 cells do not express SV40 Tag. The presence of Tag in the COS-1 derivative lines allows the introduced expression plasmid to replicate and provides a relative increase in the amount of receptor produced during the assay period. CV-1 cells are presently preferred because they are particularly convenient for gene transfer studies and provide a sensitive and well-described host cell system.

The above-described cells (or fractions thereof) are maintained under physiological conditions when contacted with physiologically active compound. "Physiological conditions" are readily understood by those of skill in the art to comprise an isotonic, aqueous nutrient medium at a temperature of about 37°C.

In accordance with yet another aspect of the present invention, there is provided a method to identify compounds which activate nuclear receptor activity, but substantially lack the ability to relieve the repression caused by an invention SMRT co-repressor, said method comprising:

comparing the reporter signal produced by two different expression systems in the absence and presence of test compound,

wherein said first expression system comprises a complex comprising:

a homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer, or retinoic acid receptor-retinoid X receptor heterodimer,

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a reporter, and

optionally, invention SMRT co-repressor, and

wherein said second expression system comprises a complex comprising:

a homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first expression system, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes,

the same response element-reporter combination as employed in said first expression system, and

optionally, invention SMRT co-repressor, and thereafter

selecting those compounds which provide:

a higher reporter signal upon exposure of said compound to said second expression system, relative to reporter signal in the absence of compound, and

substantially the same reporter signal upon exposure of said compound to said first expression system, relative to reporter signal in the absence of said compound,

wherein said selected compounds are capable of activating nuclear receptor activity, but substantially lacking the ability to relieve the repression caused by a SMRT co-repressor having a structure and function characteristic of, an invention SMRT co-repressor for retinoic acid and thyroid receptors.

In accordance with a still further aspect of the present invention, there is provided a method to identify compounds which relieve the repression of nuclear receptor activity caused by an invention SMRT co-repressor, and activate said receptor, said method comprising:

comparing the reporter signal produced by two different expression systems in the absence and presence of test compound,

wherein said first expression system comprises a complex comprising:

a homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer, or retinoic acid receptor-retinoid X receptor heterodimer,

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a reporter, and

optionally, invention SMRT co-repressor, and

wherein said second expression system comprises a complex comprising:

a homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first expression system, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes,

the same response element-reporter combination as employed in said first expression system, and

optionally, invention SMRT co-repressor, and thereafter

selecting those compounds which provide:

increased reporter signal upon exposure of said compound to said second expression system, relative to reporter signal in the absence of said compound, and

substantially increased reporter signal upon exposure of said compound to said first expression system, relative to reporter signal in the absence of said compound,

wherein said selected compounds are capable of relieving the repression of nuclear receptor activity caused by a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors, and activating said receptor.

In accordance with still another embodiment of the present invention, there are provided modified forms of the above-described SMRT co-repressor, including:

full length silencing mediator for retinoic acid and thyroid receptors plus GAL4 DNA binding domain,

full length silencing mediator for retinoic acid and thyroid receptors plus GAL4 activation domain,

full length silencing mediator for retinoic acid and thyroid receptors plus glutathione S-transferase (GST) tag,

and the like.

The above-described modified forms of invention SMRT co-repressor can be used in a variety of ways, e.g., in the assays described herein.

An especially preferred modified SMRT co-repressor of the invention comprises full length silencing mediator for retinoic acid and thyroid receptors plus GAL4 activation domain.

In accordance with a still further embodiment of the present invention, there is provided a method to identify compounds which disrupt the ability of an invention SMRT co-repressor to complex with nuclear receptors, without substantially activating said receptor, said method comprising:

comparing the reporter signal produced by two different expression systems in the absence and presence of test compound,

wherein said first expression system comprises a complex comprising:

a modified SMRT co-repressor as described above,

a homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer or retinoic acid receptor-retinoid X receptor heterodimer, and

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a reporter, and

wherein said second expression system comprises a complex comprising:

said modified SMRT co-repressor,

a homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first expression system, wherein said member is mutated such that it retains hormone dependent activation activity but has lost

its ability to repress basal level promoter activity of target genes, and

the same response element-reporter combination as employed in said first expression system, and thereafter

selecting those compounds which provide:

a lower reporter signal upon exposure of said compound to said first expression system, relative to reporter signal in the absence of said compound, and

substantially the same reporter signal upon exposure of said compound to said second expression system, relative to reporter signal in the absence of said compound,

wherein said selected compounds are capable of disrupting the ability of a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors to complex with nuclear receptors, without substantially activating said receptor.

Mutant receptors contemplated for use in this embodiment of the present invention include RAR403 homodimers, RAR403-containing heterodimers, TR160 homodimers, TR160-containing heterodimers, and the like.

Suitable host cells for use in this embodiment of the present invention include mammalian cells as well as yeast cells. Yeast cells are presently preferred because they introduce no background since SMRT (i.e., silencing mediator (SMRT co-repressor) for retinoic acid receptor (RAR) and thyroid hormone receptor (TR)) is not endogenous to yeast.

In accordance with yet another embodiment of the present invention, there is provided a method to identify compounds which activate nuclear receptor

activity, but substantially lack the ability to disrupt a complex comprising a nuclear receptor and an invention SMRT co-repressor, said method comprising:

comparing the reporter signal produced by two different expression systems in the absence and presence of test compound,

wherein said first expression system comprises a complex comprising:

a modified SMRT co-repressor as described above,

a homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer or retinoic acid receptor-retinoid X receptor heterodimer, and

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a reporter, and

wherein said second expression system comprises:

said modified SMRT co-repressor,

a homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first expression system, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes, and

the same response element-reporter combination as employed in said first expression system, and thereafter

selecting those compounds which provide:

a higher reporter signal upon exposure of said compound to said second expression system, relative to reporter signal in the absence of compound, and

substantially the same reporter signal upon exposure of said compound to said first expression system, relative to reporter signal in the absence of compound,

wherein said selected compounds are capable of activating nuclear receptor activity, but substantially lack the ability to disrupt the complex of an invention SMRT co-repressor.

Suitable host cells for use in this embodiment of the present invention include mammalian cells as well as yeast cells. Yeast cells are presently preferred because they introduce no background since SMRT is not endogenous to yeast.

In accordance with a still further embodiment of the present invention, there is provided a method to identify compounds which activate a nuclear receptor, and disrupt the ability of an invention SMRT co-repressor to complex with said receptor, said method comprising:

comparing the reporter signal produced by two different expression systems in the absence and presence of test compound,

wherein said first expression system comprises a complex comprising:

a modified SMRT co-repressor as described above,

a homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer or retinoic acid receptor-retinoid X receptor heterodimer, and

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a reporter, and

wherein said second expression system comprises a complex comprising:

said modified SMRT co-repressor,

the same homodimeric or heterodimeric member of the nuclear receptor superfamily as employed in said first expression system, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes, and

the same response element-reporter combination as employed in said first expression system, and thereafter

selecting those compounds which provide:

a reduction in reporter signal upon exposure of compound to said first expression system, relative to reporter signal in the absence of said compound, and

increased reporter signal upon exposure of compound to said second expression system, relative to reporter signal in the absence of said compound,

wherein said selected compounds are capable of activating a nuclear receptor and disrupting a complex comprising nuclear receptor and a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors.

Suitable host cells for use in this embodiment of the present invention include mammalian cells as well as yeast cells. Yeast cells are presently preferred because they introduce no background since SMRT is not endogenous to yeast.

In accordance with yet another aspect of the present invention, there is provided a method to identify compounds which activate a nuclear receptor and/or disrupt the ability of an invention SMRT co-repressor to complex with said receptor, said method comprising:

comparing the reporter signals produced by a combination expression system in the absence and presence of test compound,

wherein said combination expression system comprises:

a first homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer, or retinoic acid receptor-retinoid X receptor heterodimer,

a second homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first homodimer or heterodimer, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes (i.e., provides basal level expression),

wherein either said first homodimer (or heterodimer) or said second homodimer (or heterodimer) is operatively linked to a GAL4 DNA binding domain,

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a first reporter,

a GAL4 response element, wherein said response element is operatively linked to a second reporter, and

optionally a SMRT co-repressor of nuclear receptor activity, said SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors, and thereafter

identifying as capable of relieving the repression of nuclear receptor activity caused by a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors, but substantially lacking the ability to activate nuclear receptor activity those compounds which provide:

a higher reporter signal from the reporter responsive to the first member upon exposure of said compound to said first member, relative to reporter signal in the absence of said compound, and

substantially the same reporter signal from the reporter responsive to the second member upon exposure of said compound to said second member, relative to reporter signal in the absence of said compound, or

identifying as capable of activating nuclear receptor activity, but substantially lacking the ability to relieve the repression caused by a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors those compounds which provide:

a higher reporter signal from the reporter responsive to the second member upon exposure of said compound to said second member, relative to reporter signal in the absence of compound, and

substantially the same reporter signal from the reporter responsive to the first member upon exposure of said compound to said first member, relative to reporter signal in the absence of said compound, or

identifying as capable of relieving the repression of nuclear receptor activity caused by a SMRT co-repressor having a structure and function characteristic of

the silencing mediator for retinoic acid and thyroid receptors, and activating said receptor those compounds which provide:

a higher reporter signal from the reporter responsive to the second member upon exposure of said compound to said second member, relative to reporter signal in the absence of said compound, and

a greater increase in reporter signal from the reporter responsive to the first member upon exposure of said compound to said first member, relative to reporter signal in the absence of said compound.

Thus, the change in expression level of the two different reporters introduced in a single transfection can be monitored simultaneously. Based on the results of this single transfection, one can readily identify the mode of interaction of test compound with the receptor/SMRT complex.

Exemplary GAL4 response elements are those containing the palindromic 17-mer:

5'-CGGAGGACTGTCCTCCG-3' (SEQ ID NO:3),

such as, for example, 17MX, as described by Webster et al., in *Cell* **52**:169-178 (1988), as well as derivatives thereof. Additional examples of suitable response elements include those described by Hollenberg and Evans in *Cell* **55**:899-906 (1988); or Webster et al. in *Cell* **54**:199-207 (1988).

In accordance with still another embodiment of the present invention, there is provided a method to identify compounds which activate a nuclear receptor and/or disrupt the ability of an invention SMRT co-repressor to complex with said receptor, said method comprising:

comparing the reporter signals produced by a combination expression system in the absence and presence of test compound,

wherein said combination expression system comprises:

a modified SMRT co-repressor as described above,

a first homodimeric or heterodimeric member of the nuclear receptor superfamily selected from thyroid hormone receptor homodimer, thyroid hormone receptor-retinoid X receptor heterodimer, retinoic acid receptor homodimer, or retinoic acid receptor-retinoid X receptor heterodimer,

a second homodimeric or heterodimeric form of the same member of the nuclear receptor superfamily as employed in said first homodimer or heterodimer, wherein said member is mutated such that it retains hormone dependent activation activity but has lost its ability to repress basal level promoter activity of target genes,

wherein either said first homodimer (or heterodimer) or said second homodimer (or heterodimer) is operatively linked to a GAL4 DNA binding domain,

a response element for said member of the nuclear receptor superfamily, wherein said response element is operatively linked to a first reporter,

a GAL4 response element, wherein said response element is operatively linked to a second reporter, and thereafter

identifying as capable of disrupting the ability of a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors to complex with a nuclear receptor, without substantially activating nuclear receptor, those compounds which provide:

a lower reporter signal from the reporter responsive to the first member upon exposure of said compound to said first member, relative to reporter signal in the absence of said compound, and

substantially the same reporter signal from the reporter responsive to the second member upon exposure of said compound to said second member, relative to reporter signal in the absence of said compound, or

identifying as capable of activating nuclear receptor activity, but substantially lacking the ability to disrupt a complex comprising a nuclear receptor and a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors, those compounds which provide:

a higher reporter signal from the reporter responsive to the second member upon exposure of said compound to said second member, relative to reporter signal in the absence of compound, and

substantially the same reporter signal from the reporter responsive to the first member upon exposure of said compound to said first member, relative to reporter signal in the absence of said compound, or

identifying as capable of disrupting a complex comprising a nuclear receptor and a SMRT co-repressor having a structure and function characteristic of the silencing mediator for retinoic acid and thyroid receptors, and activating said receptor those compounds which provide:

a reduction in reporter signal from the reporter responsive to the first member upon exposure of said compound to said first member, relative to reporter signal in the absence of said compound, and

increased reporter signal from the reporter responsive to the second member upon exposure of said compound to said second member, relative to reporter signal in the absence of said compound.

In accordance with a still further aspect of the present invention, there is provided a method to identify compounds which relieve the repression of nuclear

receptor activity caused by an invention SMRT co-repressor, said method comprising determining the effect of adding test compound to an expression system comprising:

a modified member of the nuclear receptor superfamily, wherein said modified member contains an activation domain which renders said receptor constitutively active,

a fusion protein comprising the receptor interaction domain of SMRT operatively linked to the GAL4 DNA binding domain, and

a GAL4 response element operatively linked to a reporter.

Prior to addition of an effective ligand for the member of the nuclear receptor superfamily employed herein, the association of the modified member and the fusion protein will be effective to bind the GAL4 response element and activate transcription of the reporter. The presence of an effective ligand is indicated by a reduction of reporter signal upon exposure to ligand, which disrupts the interaction of the modified member and fusion protein.

Activation domains contemplated for use in the practice of the present invention are well known in the art and can readily be identified by the artisan. Examples include the GAL4 activation domain, BP64, and the like.

To summarize, a novel family of nuclear receptor SMRT co-repressor which mediates the transcriptional silencing of RAR and TR has been identified. This discovery is of great interest because transcriptional silencing has been shown to play an important role in development, cell differentiation and the oncogenic activity of v-erbA (Baniahmad et al., *EMBO J.* **11**:1015-1023 (1992)); Gandrillon et al., *Cell* **49**:687-697 (1989)); Zenke et al., *Cell* **61**:1035-1049 (1990); Barlow et al., *EMBO J.* **13**:4241-4250 (1994); Levine and Manley, *Cell* **59**:405-408 (1989); Baniahmad et al., *Proc. Natl. Acad. Sci. USA* **89**:10633-10637 (1992b); and Saitou et al., *Nature* **374**:159-162 (1995)). In fact, v-erbA mutants that harbor the Pro160->Arg change in the TR neither repress basal

transcription nor are capable of oncogenic transformation (Damm and Evans, (1993), *supra*).

The function of SMRT as a silencing mediator (co-repressor) of RAR and TR is analogous to mSin3 in the Mad-Max-Sin3 ternary complex (Schreiber-Agus et al., *Cell* **80**:777-786 (1995); and Ayer et al., *Cell* **80**:767-776 (1995)). Because GAL-SMRT functions as a potent repressor when bound to DNA, it is reasonable to speculate that the function of the unliganded receptors is to bring with them SMRT to the template via protein-protein interaction. Thus, the repressor function is intrinsic to SMRT as opposed to the TR or RAR itself (Baniahmad et al., *Proc. Natl. Acad. Sci. USA* **90**:8832-8836 (1993); and Fondell et al., *Genes Dev* **7**:1400-1410 (1993)). It is demonstrated herein that the ligand triggers a dissociation of SMRT from the receptor, which would lead to an initial step in the activation process. This would be followed (or be coincident) with an induced conformational change in the carboxy-terminal transactivation domain (c, also called AF-2), allowing association with co-activators on the transcription machinery (Douarin et al., *EMBO J.* **14**:2020-2033 (1995); Halachmi et al., *Science* **264**:1455-1458 (1994); Lee et al., *Nature* **374**:91-94 (1995); and Cavailles et al., *Proc. Natl. Acad. Sci. USA* **91**:10009-10013 (1994)). Thus, as has previously been suggested (Damm and Evans, (1993), *supra*), the ligand dependent activation of TR would represent two separable processes including relief of repression and net activation. The isolation of SMRT now provides a basis for dissecting the molecular basis of trans-repression.

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1

Isolation of SMRT

Using a GAL4 DBD-RXR fusion protein (see, for example, USSN 08/177,740, incorporated by reference herein in its entirety) as a bait in a yeast

two-hybrid screening system (Durfee et al., (1993), *supra*), several cDNA clones encoding receptor interacting proteins were isolated. One of these proteins, SMRT, interacts strongly with unliganded RAR and TR but only weakly with RXR or other receptors in yeast. This protein was selected for further characterization.

Example 2

Far-western blotting procedure

Total bacteria extracts expressing GST fusions of hRARα (aa 156-462) or hRXRα LBD (aa 228-462) and control extracts expressing GST alone or GST-PML fusion protein were subjected to SDS/PAGE and electroblotted onto nitrocellulose in transfer buffer (25 mM Tris, pH 8.3/ 192 mM glycine/ 0.01% SDS). After denaturation/renaturation from 6 M to 0.187 M guanidine hydrochloride in HB buffer (25 mM HEPES, pH 7.7/25 mM NaCl/5 mM $MgCl_2$/1 mM DTT) filters were saturated at 4°C in blocking buffer (5% milk, then 1% milk in HB buffer plus 0.05% NP40). *In vitro* translated ^{35}S-labeled proteins were diluted into H buffer (20 mM Hepes, pH 7.7/75 mM KCl/0.1 mM EDTA/2.5 mM $MgCl_2$/0.05% NP40/ 1% milk/1 mM DTT) and the filters were hybridized overnight at 4°C with (1 μM) or without ligand. After three washes with H buffer, filters were dried and exposed for autoradiography or quantitated by phosphoimager.

GST-SMRT is a GST fusion of the C-SMRT encoded by the yeast two hybrid clone. GST-SMRT has been purified, but contains several degradation products.

For yeast two-hybrid screening, a construct expressing the GAL4 DBD-hRXRα LBD (aa 198-462) fusion protein was used to screen a human lymphocyte cDNA library as described (Durfee et al., (1993), *supra*). Full length SMRT cDNA was isolated from a human HeLa cDNA library (Clontech) using the two-hybrid insert as a probe.

Using the above-described far-western blotting procedure, ^{35}S-labeled SMRT preferentially complexes with bacterial extracts expressing the RAR, marginally associates with RXR and shows no association with control extracts. In contrast, ^{35}S-PPAR selectively associates with its heterodimeric partner, RXR, but not with RAR. In a similar assay, ^{35}S-labeled RAR or TR interacts strongly with SMRT and their heterodimeric partner, RXR, but not with degraded GST products, while ^{35}S-RXR interacts only weakly with SMRT. Binding of ligand to RAR or TR reduces their interactions with SMRT but not with RXR, while binding of ligand to RXR has only slight effect. Figure 1 shows the quantitation of a dose-dependent dissociation of SMRT from RAR or TR by all-*trans* retinoic acid (atRA) or thyroid hormone (triiodothyronine or T3), demonstrating that the amount of ligand required for 50% dissociation in both cases are close to the kds for both ligands (Munoz et al. *EMBO J.* **7**:155-159 (1988); Sap et al., *Nature* **340**:242-244 (1989); and Yang et al., *Proc. Natl. Acad. Sci. USA* **88**:3559-3563 (1991)).

Full length SMRT encodes a polypeptide of 1495 amino acids rich in proline and serine residues (see Figure 2 and SEQ ID NO:1). Genbank database comparison reveals similarity of the C-terminal domain of SMRT to a partial cDNA encoding another receptor interacting protein, RIP13 (Seol et al., (1995), *supra*), whose role in receptor signaling is unknown. Within this region, there can be identified several potential heptad repeats which might mediate protein-protein interaction with the "a-helical sandwich" structure (Bourguet et al., *Nature* **375**:377-382 (1995)) of the ligand binding domain (LBD) of receptors.

Example 3

Characterization of SMRT

Unlike other nuclear receptors, unliganded RAR and TR possess a strong silencing domain which represses basal level promoter activity of their target genes (Damm et al., *Nature* **339**:593-597 (1989); Brent et al., *New Biol.* **1**:329-336 (1989); Baniahmad et al., *Cell* **61**:505-514 (1990); and Baniahmad et al., *EMBO J.*

11:1015-1023 (1992)). The preferential interaction of SMRT with RAR and TR in the absence of hormone suggests that SMRT may play a role in mediating the transcriptional silencing effect of the receptor.

To further investigate the involvement of SMRT in silencing, the interaction of SMRT with mutant receptors which display distinct silencing and/or transactivation activities was tested as follows. ^{35}S-methionine labeled receptors were used as probes to hybridize immobilized GST-SMRT in the presence (10 μM) or absence of all-*trans* retinoic acid (atRA). The total bacteria extract expressing GST-RXR was included as a control.

When quantitated by phosphoimager, RAR403 shows a 4-fold better interaction with SMRT than wild type RAR. Both full length RAR or a deletion mutant expressing only the ligand binding domain (LBD, referred to as ΔΔR) associate with SMRT; this association is blocked by ligand.

These results confirm that the LBD alone is sufficient in the interaction. The carboxy-terminal deletion mutant RAR403 is a potent dominant negative repressor of basal level promoter activity of RAR target genes (Damm et al., *Proc. Natl. Acad. Sci. USA* **90**:2989-2993 (1993); Tsai and Collins, *Proc. Natl. Acad. Sci. USA* **90**:7153-7157 (1993); and Tsai et al., *Genes Dev* **6**:2258-2269 (1992)). As might be predicted from the above studies, RAR403 and its amino terminal deletion derivative, R403, interact strongly with SMRT in either the presence or absence of ligand, consistent with SMRT mediating the repressor activity of this mutant.

Example 4

Interaction of SMRT with TR Mutants

The interaction of SMRT with two different classes of TR mutants was analyzed next. The first mutant employed is the naturally occurring oncogene, v-erbA, which has strong silencing ability but no transactivation activity (Sap et al., (1989),

supra; Sap et al., *Nature* **324**:635-640 (1986); Weinberger et al., *Nature* **318**:670-672 (1985); and Weinberger et al., *Nature* **324**:641-646 (1986)). The second mutant employed is a single amino acid change (Pro 160 -> Arg) of the rTRa (TR160) which has previously been shown to lose its capacity in basal level repression but retains hormone dependent transactivation (Thompson et al., *Science* **237**:1610-1614 (1987); and Damm and Evans, *Proc. Natl. Acad. Sci. USA* **90**:10668-10672 (1993)). If SMRT is involved in silencing, it would be expected that SMRT should interact with the v-erbA, but show little or no association with the silencing-defective TR160 mutant.

Interaction of the oncogenic v-erbA and rTRα R160 mutant (TR160) with GST-SMRT was determined in a far-western assay as described above (see Example 2). When quantitated by phosphoimager, the v-erbA shows an 18-fold better interaction with SMRT than hTRβ, and the TR160 mutant shows a 10-fold lower signal than the rTRα .

As one might expect, v-erbA interacts strongly with SMRT both in presence or absence of ligand. In contrast, full length TR160 mutant or LBD of TR160 (ΔΔTR160) does not interact significantly with SMRT when compared to the wild type receptor.

These data demonstrate that SMRT plays an important role in mediating transcriptional silencing effects of both RAR and TR. These data also suggest that the release of SMRT from receptors could be a prerequisite step in ligand-dependent transactivation by nuclear receptors.

Example 5

Formation of ternary complexes containing SMRT

RAR and TR form heterodimers with RXR, resulting in a complex with high DNA binding ability (Bugge et al., *EMBO J.* **11**:1409-1418 (1992); Yu et al., *Cell* **67**:1251-1266 (1991); and Kliewer et al., *Nature* **355**:446-449 (1992)). Since SMRT

interacts with RAR and TR, tests were conducted to determine whether SMRT can also interact with the receptor-DNA complex. Thus, the interaction of SMRT with RXR-RAR heterodimer on a DR5 element (i.e., an AGGTCA direct repeat spaced by five nucleotides) was determined in a gel retardation assay, which is carried out as follows. *In vitro* translated receptor or unprogrammed reticulocyte lysate (URL) was incubated with 1 μg of poly dIdC on ice for 15 minutes in a total volume of 20 μl containing 75 mM KCl, 7.5% glycerol, 20 mM Hepes (pH 7.5), 2 mM DTT and 0.1% NP-40, with or without ligand (in the range of about 10-100 nM employed). A ^{32}P labeled, double stranded oligonucleotide probe was added into the binding reaction (10,000 cpm per reaction), and the reaction was further incubated for 20 minutes at room temperature. The protein-DNA complex was separated on a 5% native polyacrylamide gel at 150 volts.

SMRT is seen to form a ternary complex with the RXR-RAR heterodimer on a DNA response element in the gel retardation assay. Addition of ligand releases SMRT from this complex in a dose-dependent manner.

Similarly, SMRT is seen to form a ternary complex with the RXR-TR heterodimer on a TR response element; addition of T3 disrupts the formation of this complex.

These data demonstrate that SMRT can be recruited to DNA response elements via protein-protein interaction with RAR or TR in the absence of hormone. Binding of hormone disrupts receptor-SMRT interaction and releases SMRT from the receptor-DNA complex.

Example 6

Transient transfection assay

CV-1 cells were plated in 24 well plates at a density of 50,000 cells per well. Expression plasmids were transfected into cells by lipofection using DOTAP. In

each transfection, 5 ng of GAL-RAR and 15 ng of v-erbA or SMRT were used together with 150 ng of reporter construct containing 4 copies of GAL4 binding sites in front of a minimal thymidine kinase promoter and a CMX-β-gal construct as an internal control. The relative luciferase activity was calculated by normalizing to the β-gal activity.

Example 7

Reversal of transcriptional silencing

Recently, it has been shown that over expression of RAR or TR could reverse the transcriptional silencing effect of the GAL4 DBD fusion of TR (GAL-TR) or RAR (GAL-RAR) (Baniahmad et al., *Mol Cell Biol* **15**:76-86 (1995); and Casanova et al., *Mol Cell Biol* **14**:5756-5765 (1994)), presumably by competition for a limiting amount of a SMRT co-repressor. A similar effect is observed herein when over expression of v-erbA or RAR403 mutants are shown to reverse the silencing effect of GAL-RAR and GAL-TR on the basal activity of a luciferase reporter (see Figure 3A and 3B).

In principle, over expression of SMRT should restore repressor activity when co-expressed with v-erbA or RAR403 competitors. Indeed, results presented in Figure 3C show that both the full length and the C-terminal domain of SMRT (C-SMRT) can titrate out v-erbA or RAR403 competitor activity and re-endow GAL-RAR and GAL-TR with silencing activity. In contrast, neither v-erbA nor SMRT show any effect on the transactivation activity of GAL-VP16 fusion. Thus, SMRT is able to block the titration effect of v-erbA and RAR403 and functionally replaces the putative SMRT co-repressor in this system.

Example 8

Direct recruitment of SMRT to a heterologous promoter

If SMRT is the mediator of transcription silencing of TR and RAR by interaction with template-bound unliganded receptors, then direct recruitment of SMRT

to a heterologous promoter should result in repression of basal level activity. This was tested by fusing full length SMRT to the GAL4 DBD (GAL-SMRT). The effect of the resulting fusion protein on the activity of the thymidine kinase promoter containing four GAL4 binding sites was analyzed. Figure 3D shows that GAL-SMRT, like GAL-TR, can silence basal promoter activity in a dose-dependent manner. In contrast, GAL-RXR shows no repression.

These data suggest that SMRT, when recruited to a promoter by direct DNA binding or via association with an unliganded receptor, functions as a potent transcriptional repressor.

<u>Example 9</u>

<u>Cloning Of Human And Mouse SMRT co-repressors</u>

This example describes the cloning of a full length human silencing mediator of retinoic acid and thyroid hormone receptor (SMRT co-repressor) and of two mouse SMRT isoforms, m-SMRTα and m-SMRTβ .

An examination of the previously described human SMRT co-repressor revealed that the first eight amino acids and upstream sequences were derived from a portion of ribonucleoprotein K sequence. Accordingly, a mouse spleen cDNA lambda ZAP II library (Stratagene; La Jolla CA) was screened at low stringency with a probe corresponding to approximately the 5' 1,000 base pairs (bp) of the previously identified human SMRT (s-SMRT). A 3.5 kilobase (kb) cDNA fragment was obtained that contained a unique sequence in addition to known s-SMRT sequence. The 5' end of this cDNA, and subsequently obtained clones, was used in successive rounds of screening of the mouse spleen cDNA library and a mouse brain cDNA library (Stratagene) and the full-length SMRTα isoform cDNA (SEQ ID NO: 6) and SMRTα isoform cDNA (SEQ ID NO: 10) were obtained. The mouse SMRT (m-SMRT) 5' sequence then was used at low stringency to screen a human pituitary cDNA library (Stratagene) to obtain the full-length human SMRT (h-SMRT) cDNA (SEQ ID NO: 1). All cDNA clones were

sequenced on both strands using standard methods, and have been deposited with GenBank as Accession No. AF103003 (h-SMRT; SEQ ID NOS: 3 and 5); Accession No. 113001 (m-SMRTα ; SEQ ID NOS: 6 and 7); and Accession No. 113002 (m-SMRTβ ; SEQ ID NOS: 8 and 9).

By sequentially shifting between the mouse spleen and mouse brain cDNA libraries, several clones containing a potential starting methionine and 5' untranslated region sequences were obtained. The complete polypeptide sequences of m-SMRT (SEQ ID NO: 7) and h-SMRT (SEQ ID NO: 5) are provided. In addition, a splice variant isolated from the mouse brain cDNA library encoded an m-SMRT co-repressor containing a deletion of amino acids 36 to 254 of SEQ ID NO: 7 (see SEQ ID NO: 3). The two m-SMRT co-repressors are designated SMRTα (SEQ ID NO: 7) and SMRTβ (SEQ ID NO: 9). Based on sequence similarity to N-CoR (see below), this deletion in m-SMRTβ removes the majority of the sequence in h-SMRT and m-SMRTα that is homologous to N-CoR repression domain 1 (RD1), including a portion of the Sin3A binding region.

The cloned h-SMRT (SEQ ID NO: 3) encodes a polypeptide that contains an additional 1130 amino acids at the amino terminus as compared to the previously described human SMRT co-repressor. The full length h-SMRT shares 84% identity with m-SMRTα . A comparison of h-SMRT (SEQ ID NO: 5) and N-CoR (SEQ ID NO: 11) revealed that the N-terminal extension of h-SMRT (amino acids 1 to 1030) and N-CoR (amino acids 1 to 1031) share approximately 41% identity, which is somewhat higher that the 36% identity shared between the full length proteins. However, regions within the N-CoR and SMRT N-termini share striking homology (Figures 4A and 4B).

Amino acids 1 to 160 of N-CoR are moderately conserved in h-SMRT (and m-SMRTα), sharing about 36% identity. This region of N-CoR has been reported to interact with Siah2 (Zhang et al., (1998), *supra*) and, similarly, can be involved in an

interaction of Siah2 with h-SMRT or m-SMRTα . In particular, highly conserved sequences in this region can be the specific Siah2 interaction sites (see Figure 4A).

A 52 amino acid segment from N-CoR (amino acids 255 to 312) mediates an interaction with Sin3A (Heinzel et al., *Nature* **387**:43-48 (1997)), and was presumed to represent the core of the larger RD1 region (Horlein et al., (1995), *supra*). This small interaction domain is highly conserved (about 83% identity) in h-SMRT, and the overall identity shared between SMRT and N-CoR RD1 is about 57%.

Amino acids 312 to 668 of N-CoR also are well conserved (66% identity) in h-SMRT (and m-SMRTα), and two internal blocks of sequences in this region share even greater similarity (see Figure 1B; shaded regions). These blocks are homologous to each other and to part of the SANT domain, which was identified in the yeast chromatin remodeling factor, SWI3, the yeast adapter protein, ADA2, the basal transcription factor TFIIIB, and other proteins (Aasland et al., *Trends Biochem. Sci.* **21**:87-88 (1996)), suggesting that these domains share a common and important function. The amino acids of N-CoR RD2 (see Horlein et al., (1995) *supra*) are the least conserved in h-SMRT, sharing about 30% identity.

These results demonstrate that isoforms of SMRT co-repressors are expressed in cells, as exemplified by m-SMRTα and m-SMRTβ . In addition, the results demonstrate that the previously undescribed amino terminus of SMRT co-repressors shares regions of substantial homology with N-CoR, and regions of homology are identified that indicate these sequences can mediate previously uncharacterized functions.

Example 10

Expression And Chromosomal Localization Of Smrt Co-Repressors

This example describes the tissue distribution of SMRT RNA and the chromosomal localization of human SMRT.

Total RNA was prepared from adult CB6F1 mouse tissues using TRIZOL reagent (GIBCO/BRL), and poly(A) RNA was purified from total RNA using an OLIGOTEX mRNA Kit (Qiagen, Valencia, CA). RNA was separated on 1.25% agarose/6% formaldehyde gels and transferred to a NYTRAN membrane (Scheicher & Schuell). A 720 bp m-SMRT/PstI fragment was used as a probe. Following hybridization with the SMRT probe, the filters were stripped and hybridized with a murine glyceraldehyde-3-phosphate dehydrogenase cDNA probe to allow normalization for RNA loading.

Chromosomal localization of SMRT was determined by fluorescence in situ hybridization using the 5.3 kb h-SMRT cDNA clone. The probe was labeled by nick-translation with biotin-11-dUTP, then hybridized to normal male human metaphase chromosomes. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Chromosome identification was carried out by computer inversion of the gray scale DAPI image on a PSI Imaging System (Perceptive Scientific Instruments; League City TX). Chromosome 12 confirmation was carried out using a chromosome 12-specific alpha satellite probe (Vysis; Downers Grove IL).

Previous studies using the short human SMRT co-repressor suggested that SMRT was expressed ubiquitously in various tissues. To confirm this result, expression of the full length m-SMRT was determined by northern blot analysis by using a probe consisting of nucleotides 2760 to 3620 of m-SMRT (SEQ ID NO: 6). The expression pattern was ubiquitous, as previously described, although higher levels were detected in lung, spleen, and brain. Similarly, h-SMRT was expressed ubiquitously as determined using a multiple tissue blot (CLONTECH; Palo Alto CA). It is noteworthy that two isoforms of SMRT were present in the majority of the mouse tissues and likely correspond to the m-SMRTα and m-SMRTβ isoforms.

The chromosomal location of the h-SMRT and N-CoR genes was mapped. The h-SMRT clone hybridized to the q arm of one of the C group

chromosomes. Computer-mediated banding of the DAPI stained chromosomes identified the labeled chromosome as chromosome 12, band q24. The chromosome 12 localization was confirmed by cohybridization of SMRT and a chromosome 12 alpha satellite probe, D12Z3 (Vysis), which labels the pericentromeric region of chromosome 12. The location for the human N-CoR gene was determined through a mapped human bacterial artificial chromosome clone, hCIT529I10, which is 158 kb of genomic N-CoR and resides on chromosome 11p11.2. The SMRT and N-CoR chromosomal locations can be accessed through GENEMAP98 from the Human Genome Project at http://www.ncbi.nlm.nih.gov/genemap.

These results demonstrate that the full length SMRT co-repressors and the SMRT co-repressors are expressed in various tissues. The results also demonstrate that the human SMRT gene is located on chromosome 12.

Example 11
Functional Characterization Of SMRT Amino Terminus Domains

This example demonstrates that various domains of the SMRT amino terminus can repress nuclear receptor transcriptional activity.

Experiments were performed using the plasmids pCMX-GAL4 DBD and pMH100-TK-luc (Nagy et al., (1997), *supra*). Standard PCR amplifications were used to generate GAL4 fusion constructs. All constructs were verified by double-stranded sequencing to confirm identity and reading frame.

Monkey CV-1 cells were grown in DMEM supplemented with 10% resin-charcoal stripped fetal bovine serum (FBS), 50 units/ml of penicillin G, and 50 μg/ml of streptomycin sulfate at 37°C in 7% CO_2. V-1 cells (60-70% confluence, 48-well plate) were cotransfected with 16 ng of pCMX-GAL4, 100 ng of pMH100-TK-luc, and 100 ng of pCMX-β galactosidase in 200 μl of DMEM containing 10% super-

stripped fetal calf serum (FCS) by the N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP)-mediated procedure (Nagy et al., (1997), *supra*). The amount of DNA in each transfection was kept constant by addition of pCMX. After 24 hr, the medium was replaced; cells were harvested and assayed for luciferase activity 36 to 48 hr after transfection. Luciferase activity was normalized by the level of β-galactosidase activity. Each transfection was performed in triplicate and repeated at least three times.

Based on the high degree of identity between regions of the SMRT amino terminus and the corresponding N-CoR region, the ability of regions in the SMRT amino terminus to act in transcriptional repression was examined. A nested series of nucleotide sequences encoding portions of the SMRT amino terminus fused to the GAL4 DNA binding domain (GAL-DBD) was prepared in mammalian expression vectors (Figure 5A). The constructs were cotransfected with a GAL4-TK-luciferase reporter plasmid to determine the regulatory properties of the GAL4-SMRT fusions. Repression was determined relative to the basal activity of the reporter in the presence of the GAL-DBD alone.

The entire SMRT amino terminus region (GAL4-SMRT(1-1031)) demonstrated the greatest amount of repression (approximately 38-fold), and virtually extinguished reporter activity. In comparison, GAL4-SMRT (1-303), which is equivalent to N-CoR RD1, demonstrated 6-fold repression; and GAL4-SMRT (736-1031), which is equivalent to N-CoR RD2, demonstrated about 2.6-fold repression. Surprisingly, the highly conserved SANT domain conferred a significant amount of repression (about 3.3-fold).

A smaller region (amino acids 845 to 986) within the RD2 homology region shows a higher level of sequence conservation as compared to the entire RD2 region. Deletion constructs were generated to determine whether this minimal region was sufficient for the repression activity of RD2. Deletion of flanking amino acids 736 to 845 or of amino acids 987 to 1055 did not affect the level of repression, demonstrating

that the repressor function of RD2 is contained within a 141 amino acid core sequence of RD2.

Based on sequence similarity to N-CoR, the deletion of amino acids 36 to 254 in the m-SMRTβ isoform removes the majority of RD1, including a portion of the Sin3A binding region. The effect of this deletion on SMRT function was examined by cotransfection experiments comparing repression by SMRTα to SMRTβ. These experiments revealed that SMRTβ has substantially less repressor activity than SMRTα. A construct containing the entire amino terminus of m-SMRTβ (amino acids 1 to 813) repressed activity about 2.6 fold, as compared to m-SMRTα amino acids 1 to 1031, which repressed activity about 38.1-fold. In addition, a GAL4 construct containing m-SMRT amino acids 1 to 83 repressed activity only about 1.4-fold. These results indicate that alternative splicing can add further diversity to expand the function of SMRT gene products.

Example 12

Yeast Two-Hybrid Screen and Assays

To investigate whether repression by EcR in CV-1 cells is mediated by its association with a vertebrate corepressor and whether such an interaction, if it does occur, is impaired by the A483T mutation, a mammalian two-hybrid assay with Gal4-c-SMRT was conducted.

A yeast two-hybrid screen (Fields and Song, *Nature*, **340**:245-246, (1989)) was performed by transforming approximately 2 X 10^6 Y190 yeast cells with a pAS-EcR construct and a Drosophila (0-8 hr) embryonic c-DNA two-hybrid library (Yu et al., *Nature*, **385**:552-555, (1997)). Transformants were selected onto DO-Leu-Trp-His plates containing 40 mM 3-aminotriazole (Sigma) for 3-4 days. Surviving yeast colonies were picked as primary positives and restreaked on selection plates to isolate single clones. Activation domain plasmids were rescued from the selected positive transformants for further analysis. Each clone was evaluated by testing its

potential interaction with several other nuclear receptors using the yeast two-hybrid assays. E52 was isolated and further pursued based on this selection criterion. Quantitative liquid assay of β-galactosidase was performed on positive clones 16 hr after treating the yeast cells with no ligand, or with 3 μM ligand.

pAS-EcR is a fusion gene with the region corresponding to amino acids 223-878 of EcRB1 fused C-terminally to the Gal4-DBD of the pAS1-CYH2 construct (Durfee et al., (1993), *supra*); other Gal4-DBD-based nuclear receptor constructs used in this yeast two-hybrid assay include: USP (amino acids 50-508), hRAR (amino acids 186-462) and hTR (amino acids 121-410) (Schulman et al., (1995), *supra*), and SMRT (Chen and Evans, (1995), *supra*). β-galactosidase activities were quantified by liquid assay for yeast cells treated either without ligand or with 3 μM of corresponding hormone. All-trans retinoic acid (ATRA) is a ligand of RAR; 3,3',5-triiodothyroacetic acid (T3) is a ligand of TR.

Similar yeast two-hybrid assays were also used to examine the interaction between SMRTER and mSin3A and dSin3A.

Example 12

Cloning SMRTER

To isolate full-length SMRTER cDNA, a XhoI insert fragment isolated from the E52 clone was used to screen male and female Tudor c-DNA libraries (gift of Tulle Hazelrigg). This initial screen resulted in isolating three overlapping c-DNA clones covering the region of amino acid 2094 to the C terminus of SMRTER Additional regions were obtained from three consecutive library screens using two cosmid clones isolated from the Tamkun genomic library (gift of John Tamkun). Sequences of these overlapping c-DNA and genomic clones were assembled to obtain a conceptual open reading frame of SMRTER 3446 amino acids in length (SEQ ID NO:12; Figure 8A). The translational initiation codon was designated based on the sequences that match the consensus Kozak codons and is preceded by three in-frame

consecutive stop codons in the upstream region. Both strands of the sequences of the c-DNA clones were determined using an ABI prism Big Dye® terminator cycle sequencing ready reaction kit (PE Biosystems) and ABI 377 instrument.

Example 14
Plasmids

CMV promoter-driven expression plasmids of EcR, USP, RXR, c-SMRT, β-galactosidase, and pMH100-TK-luc reporter, and yeast plasmids of RAR, TR, and SMRT have been described previously (Yao et al., (1992), *supra*, Yao et al., (1993), *supra*; Chen and Evans, (1995), *supra*; Schulman et al., (1995), *supra*; Chen et al., *Proc. Natl. Acad. Sci. USA* **93**:7567-7571, (1996); Nagy et al., (1997), *supra*). hsp27EcR-TK-Luc, a reporter with six copies of the hsp27EcRE, is a gift of Barry Forman. CMV vector-driven EcR A483T and Gal4-SMRD3 mutations were generated using the Transformer® site-directed mutagenesis kit (Clontech) with proper selection primers and the mutagenic primers that correspond to the missense mutation (A483T) of EcR and to the designated mutations, M2 and M3, in the SMRD3 domain, respectively. Other plasmids were constructed with standard techniques, including various enzyme digestions or PCR amplification.

Example 15
Cell Culture and Transfection

CV-1 cells were grown in Dulbecco's modified Eagles medium at 37°C in 5% CO_2. The media were supplemented with 10% AG1-X8 resin charcoal double-stripped calf bovine serum, 50 U/ml penicillin G, and 50 µg/ml streptomycin sulfate. Approximately 20 hr after CV-1 cells (10^5 cells) were plated in 48-well cell culture clusters (Costar), cells were transiently transfected with plasmids using DOTAP according to the instructions of the manufacturer (Boehringer Mannheim). The amount of CMV promoter-driven expression vectors, β-galactosidase gene

expression vector, CMX-lacZ, and reporter, pMH100-TK-luc or hsp27EcRE-TK-Luc, were in the range of 100-200 ng, 500 ng, and 400 ng, respectively, for six wells of each 48-well clusters in each transfection experiments. At least 4 hr after transfection, each medium was replaced with medium either without ligand, or with 1 μM of MurA. Cells were harvested and assayed approximately 48 hr after transfection. All experiments were performed in triplicate and repeated with similar results.

CV-1 cells were transfected with wild-type EcR or EcR A483T, along with vp16-USP and a reporter, hsp27EcRE-TK-Luc, which contains six copies of the hsp27EcRE fused to the thymidine kinase (TK) promoter-luciferase reporter. VP16-USP fusion contains the region of USP (amino acids 50-508) fused C-terminally to the VP16 domain. Muristerone A (MurA) is a potent ecdysone agonist (Christopherson et al., *Proc. Natl. Acad. Sci. USA,* **89**:6314-6318, (1992)). In all experiments, cells were also cotransfected with CMV-lacZ, which is used to normalize the luciferase activity. As shown in Figure 6A, the ability to dimerize with USP is reflected in reporter activity without treatment with hormone (open bar), and the ability to respond to hormone is reflected in reporter activity when cells were treated with 1 μM Muristerone A (closed bar).

CMV promoter-driven expression vector including wild-type EcR or EcR A483T was cotransfected with VP16-USP and Gal4-c-SMRT (amino acids 981 to C terminus) (Chen and Evans, (1995), *supra*) into CV-1 cells to examine its effect on the interaction with vertebrate corepressor. All cells were also cotransfected with a TK-luciferase reporter construct, pMH100-TK-Luc, containing four copies of the yeast Gal4-responsive element. EcR A483T corresponds to a single amino acid change (alanine→threonine) at the 483 site of EcR (Bender et al., (1997), *supra*). The results of this experiment (Figure 6B) show that EcR A483T disrupts the interaction with SMRT.

Example 16

In Vitro Interacting Assays

Glutathione S-transferase fusion proteins, including GST-X, GST-ERID1 (amino acids 1698-2063 of SEQ ID NO:1), and GST-ERID2 (amino acids 2951-3038 of SEQ ID NO:1), were expressed in E. coli DH5 cells, and extracts were affinity purified by binding to glutathione Sepharose 4B beads. Bound proteins used as affinity matrices in pull-down experiments were first equilibrated with the binding buffer (20 mM HEPES [pH 7.9], 150 mM NaCl, 1 mM EDTA, 4 mM MgCl2, 1 mM DTT, 0.06% NP40, 10% Glycerol, 0.25 mM PMSF, 1 mg BSA). For pull-down assays using GST-ERID1 (amino acids 1698-2063 of SEQ ID NO:1) and GST-ERID2 (amino acids 2951-3038 of SEQ ID NO:1), additional hsp27EcRE (0.05 μg/ml) was added to the binding buffer. In this experiment, 30 μl of 50% GST-protein beads slurry, containing approximately 1 μg of proteins, were incubated with 10 μl of 35S-methionine-labeled proteins in 300 μl of the binding buffer (with or without 3 μM of MurA as indicated) for 30 min at room temperature. After the incubation, beads were washed three times with the binding buffer (with or without ligand) and resuspended in SDS-PAGE sample buffer before loading. After electrophoresis, bound radio-labeled proteins were visualized by autoradiography. 35S-methionine-labeled EcR, USP were generated in a coupled transcription-translation system, TNT (Promega), using CMX-EcR (T7) and CMX-uspK (T7) constructs as templates, respectively.

Example 17

Immunohistochemistry and Immunofluorescence

Antibodies against SMRTER were raised in rabbits immunized with bacterially expressed glutathione S-transferase fusion proteins corresponding to the region (amino acids 2477-2648 of SEQ ID NO:1) of SMRTER. Specific antibodies were purified by affinity chromatography through antigen-linked columns and used at 1:200 dilution for tissue staining. Tissues for whole-mount staining were dissected at the wandering third instar stage of the Canton-S strain larvae and fixed (4%

formaldehyde in 1? PBS, 50 mM EGTA) for at least 30 min. Preincubation, secondary antibodies, washes, and peroxidase reactions are described in the protocol of the Elite ABC (Rabbit IgG) kit (Vector). For the pilot experiments, partially purified IgG from preimmunization serum was used. For polytene chromosome staining, salivary glands were dissected according to the method described in Zink et al., *EMBO J.*, **10**:153-162, (1991).

Chromosome spreads were costained with affinity-purified anti-SMRTER (1:100) polyclonal antibody and with anti-USP monoclonal antibody (ABIII/AD5; gift of F. Kafatos, 1:100 dilution). SMRTER was detected with Texas red-conjugated donkey anti-rabbit secondary antibody (1:100 dilution), and USP was detected with FITC-conjugated donkey anti-mouse secondary antibody (1:100 dilution) (Jackson ImmunoResearch Labs).

Example 18

ER Interacts Genetically with DSinA

In keeping with the evidence that dSin3A is a component in EcR regulatory pathway, an experiment was conducted to examine whether dSin3A interacts genetically with EcR using several previously characterized Drosophila EcR and dSin3A mutants (Bender et al., (1997), *supra*; Neufeld et al., (1998), *supra*). In the experiment, in which female dSin3AK07401 were crossed with male EcRE261st using techniques known in the art (see Table 1 below), only approximately 14% of the scored EcRE261st/dSin3AK07401 progenies survived, a percent that is significantly lower than the expected 33.3%. This suggests that a large portion of the EcRE261st/dSin3AK07401 flies either die prior to eclosion or fail to eclose. Additionally, surviving EcRE261st/dSin3AK07401 escapers showed delayed development and wing defects, in which wings are held horizontally at 45°-90° angle from the body axis. These results suggest that dSin3A shares an overlapping regulatory pathway with EcR.

In a reverse genetic cross, in which female EcRE261st were crossed with male dSin3AK07401, none of the EcRE261st/dSin3AK07401 flies survived to adulthood. These results suggest that EcRE261st/dSin3AK07401 results in a genetically sensitized background. When the maternally deposited EcR in embryos descended from female EcRE261st/SM6b was cut in half, the lethality for EcRE261st/dSin3AK07401 was further increased. These results reveal that, in addition to its previously known zygotic function, EcR also contributes maternally to Drosophila development.

Table 1

Table 1. EcR Interacts Genetically with DSin3A

Cross			$EcR^{E261st}/DSin3A^{KO7401}$ Surviving Rate (%)
$DSin3A^{KO7401}/CyO$ × $EcR^{261st}/SM6b$	♀ ♂		14 (n = 141)
$EcR^{261st}/SM6b$ × $DSin3A^{KO7401}/CyO$	♀ ♂		0 (n = 144)

A similar wing held-out phenotype is also observed in $EcR^{E261st}/DSin3A^{xe374}$, $Df(2R)nap11/DSin3A^{KO7401}$, and $Df(2R)nap11/Dsin2A^{xe374}$. EcR^{E261st} and Df(2R)nap11 are both described in Figure 6. $Dsin2A^{KO7401}$ is an allele with a P element insertion within the 5' intron of Sin3A. $DSin3A^{xe374}$ is an X ray-generated allele (Neufeld et al., (1998)). n=the number of surviving flies scored. Note that CyO/SM6b is lethal.

EcRA483T flies showed developmental abnormalities in wings and tergites. A similar phenotype, although with a lower penetration rate, has been also observed in EcRA483T/Df(2R)20B and in EcRA483T/Df(2R)nap11. Df(2)20B and Df(2)nap11 are both deficiencies in which EcR is deleted (Bender et al., (1997), *supra*). Sequence alignment of EcR with the vertebrate TR, RAR, and v-erbA, an oncogenic TR variant, revealed that alanine 483 is located within a highly conserved 23-amino acid (aa) loop region connecting helices 3 and 4, termed the LBD signature

motif (Wurtz et al., *Nat. Struct. Biol.*, **3**:206, (1996)) (see Figure 6C). Based on structural studies of vertebrate nuclear receptors (for review, see Moras and Gronemeyer, (1998), *supra*), this alanine residue appears to be on the exposed surface, consistent with it being a potential corepressor binding site for nuclear receptors.

These in vivo studies indicate that EcRA483T is a semilethal allele (Bender et al., (1997), *supra*). When EcRA483T is in trans with EcRE261st, an allele that removes both the DBD and LBD domains of EcR, animals are primarily lethal (>95%). The few surviving EcRA483T/EcRE261st flies, however, display significant delays in development, blistered wings, and defective tergites, indicating that EcR is involved in the development of these tissues. The ability of EcR to bind a vertebrate corepressor and the loss of this property in EcR A483T suggests that the defects observed in EcRA483T flies may result from the disruption of its interaction with an as yet unidentified Drosophila corepressor.

Example 19

Isolation of an EcR-Interacting Factor

The CMV promoter-driven expression vector including wild-type EcR or EcR A483T, was cotransfected with vp16-USP and Gal4-c-SMRT (amino acids 981 to C terminus) (Chen and Evans, (1995), *supra*) into CV-1 cells to examine its effect on the interaction of the invertebrate SMRTER with vertebrate corepressor. All cells were also cotransfected with a TK-luciferase reporter construct, pMH100-TK-Luc, containing four copies of the yeast Gal4-responsive element. EcR A483T corresponds to a single amino acid change (alanine→threonine) at the 483 site of EcR (Bender et al., (1997), *supra*). Although EcR readily interacted with SMRT in both mammalian and yeast cells (Figure 6B; Figure 7), repeated low-stringency hybridization screens failed to identify a Drosophila homolog of SMRT. No SMRT/N-CoR homolog was found in C. elegans.

Example 20

Isolation and Characterization of an

EcR-Interacting Clone - Yeast Two-hybrid screen

To pursue the isolation of an EcR corepressor, a yeast two hybrid interaction screen was performed of a Drosophila embryonic cDNA library using pAS-EcR as bait. E52 was isolated as one of the complementary positive clones from a yeast two-hybrid screen with pAS-EcR as bait, as described in Example 12.

Example 21

Characterization of a Repression-Defective EcR Allele, EcRA483T

(A) CV-1 cells were transfected with wild-type EcR or EcR A483T, along with vp16-USP and a reporter, hsp27EcRE-TK-Luc, which contains six copies of the hsp27EcRE fused to the thymidine kinase (TK) promoter-luciferase reporter. In all experiments, cells were also cotransfected with CMV-lacZ, which is used to normalize the luciferase activity. The ability to dimerize with USP was reflected in reporter activity without treatment with hormone (open bar), and the ability to respond to hormone was reflected in reporter activity when cells were treated with 1 μM Muristerone A (closed bar). vp16-USP fusion contains the region of USP (amino acids 50-508) fused C-terminally to the vp16 domain. Muristerone A (MurA) is a potent ecdysone agonist (Christopherson et al., (1992), *supra*). In these tests EcR A483T was selectively defective in repression.

(B) CMV promoter-driven expression vector including wild-type EcR or EcR A483T was cotransfected with vp16-USP and Gal4-c-SMRT (amino acids 981 to C terminus) (Chen and Evans, (1995), *supra*) into CV-1 cells to examine its effect on the interaction with vertebrate corepressor. All cells were also cotransfected with a TK-luciferase reporter construct, pMH100-TK-Luc, containing four copies of the yeast Gal4-responsive element. EcR A483T corresponds to a single amino acid change (alanine threonine) at the 483 site of EcR (Bender et al., (1997), *supra*). The

results of this test show that EcR A483T disrupts the interaction with SMRT.

(C) Sequence alignment of EcR with the vertebrate TR, RAR, and v-erbA, an oncogenic TR variant, reveals that the alanine 483 of the EcRA4831T mutant is located within a highly conserved 23-amino acid (aa) loop region connecting helices 3 and 4, termed the LBD signature motif (Wurtz et al., (1996), *supra*) (Figure 6C). Based on structural studies of vertebrate nuclear receptors (for review, see Moras and Gronemeyer, (1998), *supra*), this alanine residue appears to be on the exposed surface, consistent with it being a potential corepressor binding site for nuclear receptors.

In vivo studies indicated that EcRA483T is a semilethal allele (Bender et al., (1997), *supra*). When EcRA483T is in trans with EcRE261st, an allele that removes both the DBD and LBD domains of EcR, animals are primarily lethal (>95%). The few surviving EcRA483T/EcRE261st flies, however, display significant delays in development, blistered wings, and defective tergites, indicating that EcR is involved in the development of these tissues. The ability of EcR to bind a vertebrate corepressor and the loss of this property in EcR A483T suggested to us that the defects observed in EcRA483T flies may result from the disruption of its interaction with an as yet unidentified Drosophila corepressor.

Example 22

Isolation of an EcR-Interacting Factor

Although EcR readily interacts with SMRT in both mammalian and yeast cells (Figure 6B; Figure 7), repeated low-stringency hybridization screens failed to identify a Drosophila homolog of SMRT. Given that no SMRT/N-CoR homolog is found in C. elegans, it was believed that either a SMRT/N-CoR-like corepressor is not conserved in invertebrates or, alternatively, invertebrate corepressors may diverge significantly from their vertebrate counterparts. To pursue the isolation of an EcR corepressor, a yeast interaction screen of a Drosophila embryonic cDNA library using

EcR as bait was conducted as described in Example 19. This screen resulted in the isolation of a clone, E52, whose protein product interacts with EcR as well as with the vertebrate RAR and TR, but notably not with USP (Figure 7). Unlike the interaction between E52 and RAR, which can be dissociated by all-trans retinoic acid, the interaction between E52 and EcR, or the interaction between SMRT and EcR, is not dissociated by Muristerone A (MurA). This result suggests that other factors essential for the dissociation of E52 from EcR, such as USP, are missing in yeast (see below).

Example 23

Isolation and Characterization of an EcR-Interacting Clone

E52 was isolated as one of the complementary positive clones from a yeast two-hybrid screen. Isolation of overlapping cDNA and genomic clones led to the identification of a full-length sequence encoding a large protein of 3446 amino acids (Figure 8A). This protein contains several unusually long stretches of Gln, Ala, Gly, and Ser repeats. Comparative analysis reveals it to be a novel protein with limited regions of clear homology with the vertebrate nuclear receptor corepressors SMRT and N-CoR (Chen and Evans, (1995), *supra*; Hörlein et al., (1995), *supra*; Ordentlich et al., (1999), *supra*; Park et al., (1999), *supra*). This protein SMRTER, SMRT-related ecdysone receptor-interacting factor, was shown by Northern blot analysis to encode large transcripts (>12 kb) expressed broadly throughout the embryonic stage and three larvae stages, as well as in adult Drosophila flies.

Example 24

Molecular and Biochemical Analysis for ERID1 and ERID2

Interaction with the EcR complex was evaluated based on transient transfection with the Gal4-SMRTER fusion genes. USP, EcR-vp16 (VP16 transactivating domain was fused C-terminally to the end of the EcRB1 isoform), and the reporter, pMH100-TK-Luc.

In vitro pull down assays (Example 12) were conducted to determine whether EcR interacts with ERID1 and ERID2. In vitro translated 35S-methionine-labeled EcRB1 alone, or a mixture of 35S-methionine-labeled EcRB1 and unlabeled USP, or 35S-methionine-labeled USP alone, were incubated with GST, GST-ERID1 (amino acids 1698-2063 of SEQ ID NO:1), or GST-ERID2 (amino acids of SEQ ID NO:1). GST-ERID1 and GST-ERID2, but not GST alone, pull down labeled EcR, whereas little interaction is found between USP and any of the three GST proteins. In addition, the pull-down complex was disrupted by the addition of 3μM MurA when USP is present. These in vitro results establish that SMRTER and EcR may interact directly.

Further in vitro tests were conducted to determine ERID1, ERID2, and c-SMRT compete with each other to bind EcR. Gal4-ERID1 (amino acids 1698-2063 of SEQ ID NO:1) or Gal4-ERID2 (amino acids 2929-3181 of SEQ ID NO:1), along with EcR-vp16 and USP, were transfected in CV-1 cells as described above. In this competition experiment, additional ERID1, ERID2, and c-SMRT (Chen et al., (1996), *supra*) were cotransfected into cells. ERID1 (1698-2063) and ERID2 ((amino acids 2929-3038 of SEQ ID NO:1) were tagged with the nuclear targeting signal (MAPKKKRKV) (SEQ ID NO:3) to ensure that these proteins were localized in nuclei. As shown in Figure 11C, interaction between each Gal4-ERID fusion and EcR-vp16:USP was significantly decreased by both ERIDs and by c-SMRT. Interestingly, a more prominent effect was observed in experiments when Gal4-ERID1 (amino acids 1698-2063 of SEQ ID NO:1) was challenged by ERID2, and, conversely, a more efficient competition was achieved by ERID1 to Gal4-ERID2 (amino acids 2094-3181 of SEQ ID NO:1). Together, these results suggest that ERID1, ERID2, and c-SMRT may bind similar or overlapping surface(s) in EcR.

Example 25

SMRTER Colocalizes with the EcR on Polytene Chromosomes

SMRTER antibodies were prepared as described in Example 12 to examine its cytological and chromosomal localization patterns of SMRTER. Consistent with its action as a corepressor of EcR, SMRTER was localized to nuclei of salivary glands and of fat bodies, as well as to nuclei of eye, wing, and leg imaginal discs isolated from the third instar larvae.

Next association of SMRTER with the EcR:USP complex on chromosomes was examined. The USP staining pattern was used as an index for EcRs presence on chromosomes. Since USP and EcR colocalized with each other on polytene chromosomes (Yao et al., (1993), *supra*), chromosomal spreads prepared from the salivary glands of wandering third instar larvae (prior to pupariation) were subjected to simultaneous immunological staining with antibodies against SMRTER and USP. SMRTER was detected with antibody conjugated with Texas red, USP with FITC.

To visualize the band, interband, and puffing patterns of the polytene chromosomes, the chromosomes were counterstained with DAPI to show the banding regions while leaving the interbands and puffs unstained or lightly stained. Indirect immunofluorescence staining revealed that SMRTER is a chromosome-bound protein and colocalizes with USP (FITC) at a majority of chromosomal sites; whereas in a pilot experiment, no such staining patterns were detected using the preimmunization serum. The strongest SMRTER staining was primarily associated with the boundary between band and interband regions as well as within the interband regions of chromosomes counterstained with DAPI. This result confirms that, as an EcR-associating factor, SMRTER is recruited by the EcR:USP heterodimers to their specific target chromosomal loci.

SMRTER staining can still be detected in puffed regions, such as the 2B puff. Since the polytene chromosomes consist of a parallel arrangement of several hundred to two thousand copies of the euchromatic portions of the chromosomes, an individual binding protein like SMRTER may be cycling on and off, resulting in a steady state of signals detected in the broader chromatin regions. Whether or not SMRTER levels actually change prior to or after the peak of ecdysone pulses remains to be established.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.